# Role of NBS1 in DNA Double-Strand Break Response

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**Summary**

DNA is a stable and inert chemical but its integrity is constantly threatened by a large number of endogenous and exogenous agents. DNA double strand breaks (DSBs) are the most lethal kind of DNA damage. If DSBs are not repaired immediately and accurately, they can lead to chromosomal instability and thus corrupt the genomic integrity, which is a critical step into oncogenesis. To counter the constant threat to the genetic integrity, cells have evolved sophisticated DNA repair and signaling processes, collectively known as the DNA-damage response (DDR), to detect DNA damage, signal its presence and mediate its repair. Various studies using different experimental systems have led to the conclusion that whereas there are many variations on the precise mechanisms of DSB repair, these events can be broadly categorized into these two distinct and complementary pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ). One crucial difference between HR and NHEJ is that HR requires a homologous sequence (usually the sister chromatid) as template, whereas NHEJ does not. Moreover, in order to initiate HR, the DNA ends need to be resected. Thus, DNA-end resection is crucial for DSB repair pathway choice, as it commits cells to HR.

In vertebrates, DNA end resection is initiated by the concerted action of the MRE11-RAD50-NBS1 (MRN) complex and CtIP. The precise role of NBS1 subunit of the MRN complex in DNA end resection has not yet been described in detail. The N-terminal region of NBS1 comprises a forkhead-associated (FHA) domain and 2 BRCA1 C-terminal (BRCT) domains. These domains compose a unique bivalent phosphopeptide recognition module that modulates the activities of the MRN complex through phosphorylation-dependent protein-protein interactions.

In the first part of this PhD thesis, a new NBS1 complementation system was developed with the aim to perform detailed structure/function analysis of the role of the FHA/BRCT domains of NBS1 in DSB repair by HR. Thereby, we specifically focused on NBS1's role in DNA end resection. This analysis revealed that in response to irradiation the FHA and BRCT domains of NBS1 contribute in an additive manner to

DNA end resection during the HR branch of DSB repair. Moreover, we could gather experimental evidence suggesting that mechanistically, NBS1 may act in DNA end resection through a direct interaction between its N-terminal FHA/BRCT domain and phosphorylated CtIP.

The second part of this PhD thesis dealt with the role of the FHA/BRCT domains of NBS1 in G2M checkpoint activation and G2M checkpoint maintenance. Using our functional complementation system of NBS1 as well as a new NBS1 knockout cell line, we demonstrated that NBS1 is important for the activation of the G2M checkpoint. Similar to the situation in the HR branch of DSB repair, the FHA and BRCT domains of NBS1 contribute towards G2M checkpoint function in an additive and non-redundant manner. Surprisingly, we didn't observe any effect of NBS1 depletion on G2M checkpoint maintenance, even though G2M checkpoint maintenance has recently been linked to DNA end resection.

In summary, we conclude that NBS1 is important for DNA end resection and G2M checkpoint activation. The FHA and BRCT domains of NBS1 are critical for the overall role of NBS1 in the DDR and they act in an additive and non-redundant manner, both in DNA resection, as well as in the G2M checkpoint activation.

**Zusammenfassung**

DNA ist eine stabile und inerte chemische Substanz, aber ihre Unversehrtheit wird ständig durch eine grosse Anzahl von exogenen und endogenen Einflüssen bedroht. DNA Doppelstrangbrüche gehören zu den gefährlichsten Arten von DNA Schäden. Wenn DNA Doppelstrangbrüche nicht sofort und sehr genau repariert werden besteht die Gefahr dass sich chromosomale Instabilität ausbreitet und dadurch die genomische Integrität verloren geht, was letztlich zur Krebsentstehung beitragen kann. Um dieser Bedrohung entgegen zu wirken haben Zellen im Laufe der Evolution ausgeklügelte DNA Reparatur und Signalkaskaden entwickelt welche kollektiv als ‚DNA damage respone' (DDR) bezeichnet werden. Diese zellulären Prozesse erkennen DNA Schäden, signalisieren ihr Vorhandensein und regulieren die DNA Reparatur.

Verschiedene Untersuchungen haben zum Schluss geführt dass DNA Doppelstrangbrüche grundsätzlich durch zwei mechanistisch verschiedene Reparaturprozesse repariert werden. Homologe Rekombination (HR) und nicht-homologe Zusammenfügung der DNA Enden (‚non-homolgous end joining'; NHEJ). Ein wichtiger Unterschied zwischen diesen zwei Reparaturwegen ist der Bedarf eines homologen DNA Moleküls (vorzugsweise des Schwesterchromatids) als Matrize für die Reparatur im Falle der HR jedoch nicht bei der NHEJ. Zusätzlich bedarf HR zwingend der sogenannten DNA End-Resektion. Deshalb kann der Prozess der DNA End-Resektion als ausschlaggebend für die Auswahl des Doppelstrangbruch Reparaturwegs gesehen werden.

In Zellen von Wirbeltieren wird die DNA End-Resektion durch die gemeinsame Aktivität des MRE11-RAD50-NBS1 (MRN) Proteinkomplexes und des Faktors CtIP eingeleitet. Dabei ist die Funktion der NBS1 Untereinheit des MRN Komplexes im Prozess der DNA End-Resektion noch nicht genau verstanden. Die N-terminale Region von NBS1 ist zusammengesetzt aus einer ‚Forkhead associated' (FHA) Domäne und zwei ‚BRCA1 C-terminal' (BRCT) Domänen. Diese beiden Domänen formen ein einmaliges, bivalentes Phosphopeptid-Erkennungsmodul welches die Funktionen des MRN Komplexes durch phosphorylierungsabhängige Protein-Protein

Interaktionen moduliert. Im ersten Teil dieser Doktorarbeit wurde ein neues zelluläres Komplementierungssystem für NBS1 entwickelt mit dem Ziel detaillierte Struktur/Funktion Analysen zur Rolle der FHA/BRCT Domänen von NBS1 in der Doppelstrangbruch Reparatur durchzuführen. Dabei sollte das Augenmerk vor allem auf der Funktion von NBS1 in der DNA End-Resektion liegen. Diese Analysen zeigten dass nach der Bestrahlung von Zellen mit ionisierender Strahlung die FHA und die BRCT Domänen von NBS1 in einer additiven und nicht redundanten Art und Weise zur DNA End-Resektion während des HR DNA Reparaturwegs beitragen. Zusätzlich konnten Daten gesammelt werden die darauf hinweisen dass eine direkte Interaktion zwischen den N-terminalen FHA und BRCT Domänen von NBS1 mit phosphoryliertem CtIP die Rolle von NBS1 in der DNA-End Resektion mechanistisch erklären könnte.

Im zweiten Teil dieser Doktorarbeit wurde die Aufgabe von NBS1 und seiner FHA und BRCT Domänen in der Aktivierung und Aufrechterhaltung des G2M Checkpoints untersucht. Dazu kam neben dem oben genannten zellulären Komplementierungssystem auch eine neue NBS1 ‚knockout' Zelllinie zum Einsatz. Damit konnte gezeigt werden dass NBS1 für die Aktivierung des G2M Checkpoint eine wichtige Rolle spielt und dass in Analogie zum HR Reparaturweg die FHA und BRCT Domänen von NBS1 in einer additiven und nicht-redundanten Art und Weise zur Aktivierung des G2M Checkpoints beitragen. Überraschenderweise löste der Verlust von NBS1 keinen Defekt in der Aufrechterhaltung des G2M Checkpoints aus, obschon die Aufrechterhaltung dieses Checkpoints kürzlich mit dem Prozess der DNA End-Resektion kausativ in Zusammenhang gebracht wurde.

Zusammenfassend konnte darauf geschlossen werden dass NBS1 sowohl für den Prozess der DNA End-Resektion sowie für die Aktivierung des G2M Checkpoints wichtig ist. Dabei sind die FHA und BRCT Domänen von NBS1 kritisch für diese Aktivitäten und sie tragen in einer additiven und nicht-redundanten Art und Weise sowohl zum Prozess der DNA End-Resektion sowie zur G2M Checkpoint Aktivierung bei.

## ABBREVIATIONS:

| | |
|---|---|
| 53BP1 | p53 binding protein |
| 8-oxo-dG | 8-oxo-deoxyguanosine |
| 6-4 PPs | 6-4 photoproducts |
| Aa | Amino acid |
| AT | ataxia telangiectasia |
| ATR | ataxia telangiectasia mutated |
| BRCA1/2 | breast cancer susceptibility gene 1/2 |
| BRCT | BRCA1 C-terminus domain |
| BrdU | bromodeoxyuridine |
| Chk1/2 | checkpoint kinase 1/2 |
| CDKs | cyclin-dependent kinases |
| CIN | chromosomal instability |
| CPDS | cyclobutane pyrimidine dimers |
| CPT | camptothecin |
| CSR | class switch recombination |
| CtIP | CtBP interacting protein |
| CO | Crossover |
| DOX | doxycycline |
| DDR | DNA damage response |
| DSBs | DNA double strand breaks |
| dHJ | Double Holliday junction |
| EdU | 5-ethynyl-2’-deoxyuridine |
| EXO1 | Exonuclease 1 |
| ETOP | etoposide |
| FHA | forkhead-associated domain |
| GFP | green fluorescent protein |
| HR | homologous recombinaton |
| ICL | interstrand crosslink |
| IR | Ionizing radiation |
| LigIV | DNA ligase |
| MDA | malondialdehyde |
| MDC1 | mediator of DNA damage checkpoint 1 |
| MEFs | mouse embryonic fibroblasts |
| MMC | Mitomycin C |
| MMS | methyl methanesulfonate |
| MRE11 | meiotic recombination 11 homolog 1 |
| MRN | MRE11-RAD50-NBS1 |
| NBS1 | Nijmegen breakage syndrome |
| NCO | non crossover |

| | |
|---|---|
| NER | Nucleotide excision repair |
| NHEJ | non-homologous end-joining |
| alt-NHEJ | alternative NHEJ |
| c-NHEJ | classical NHEJ |
| PCNA | proliferating cell nuclear antigen |
| PI | propidium iodide |
| PIKK | phosphatidylinositol 3-kinase-like protein kinase |
| pRb | retinoblastoma protein |
| RAD50 | radiation sensitive 50 |
| RIF 1 | Rap-interacting factor 1 |
| RPA | replication protein A |
| RNF8 | ring finger protein 8 |
| ROS | reactive oxygen species |
| Sae2 | sporulation in absence of spo11 |
| SDSA | synthesis dependent strand annealing |
| SD | Standard deviation |
| SEM | Standard error of mean |
| SMC | structural maintenance of chromosomes |
| Spo11 | sporulation-specific protein 11 |
| SSA | single-strand annealing |
| TLS | translesion DNA synthesis |
| Top i/II | Topoisomerase i/II |
| UV | ultraviolet light |
| XLF | XRCC4-like factor |
| XRCC4 | X-ray repair cross-complementing protein 4 |
| Xrs2 | X-ray sensitive protein 2 |

# 1. Introduction

## 1.1. Genomic Instability and cancer

One of the major endeavors of a cell is to safeguard the encoded information in the genetic material, a task that is pivotal for the faithful maintenance and propagation of life. In living cells, the genetic material is DNA, in which two polynucleotide chains wrap around one another to form the famous structure, popularly known as DNA double helix. James Watson and Francis Crick discovered DNA double helix structure in 1953 and this marked a new era of modern biology. The structural organization of DNA makes it a very stable and inert chemical, however its structural integrity is under constant threat by a large number of exogenous and endogenous threats. In addition, changes that occur during physiological cellular processes like DNA repair, DNA duplication, and recombination further challenge the genomic integrity by inciting alterations in the structure of the DNA double helix. It has been estimated that each of the approx. $10^{13}$ cells in a human body experience around $10^{5}$ spontaneous lesions per cell per day (Ciccia and Elledge, 2010; Hoeijmakers, 2009).

A cell is prone to a large repertoire of genetic alterations that can range from point mutations, small insertions and deletions of nucleotides to gross chromosomal rearrangements such as translocations, duplications, inversions or deletions. These alterations also include a condition known as aneuploidy, in which the cell has one or several extra chromosomes or one chromosome is entirely missing (Draviam et al, 2004). Changes in chromosome number and structure are also referred to as chromosomal instability (CIN) (Gonzalez-Suarez et al) and it is the most common form of genomic instability in cancer cells (Negrini et al., 2010). Cancer is a complex disease, with multiple genes of diverse pathways involved in different stages of initiation, progression, invasion, and metastasis. If a normal cell acquires too many genetic alterations it can become carcinogenic in a multistep process. In fact, it is widely accepted that the sequential accumulation of mutations that activate oncogenes and disrupt tumor suppressor genes, combined with multiple cycles of clonal selection and evolution facilitate the process of carcinogenesis. Because

genetic instability increases the rate at which genetic alterations occur, chances that some cells acquire genetic alterations that provide proliferative advantage are also increased, thus paving the way to carcinogenesis. It has been estimated that disruption of about eight cellular barriers is required for the malignant transformation of cells to ensue (Hanahan and Weinberg, 2011).

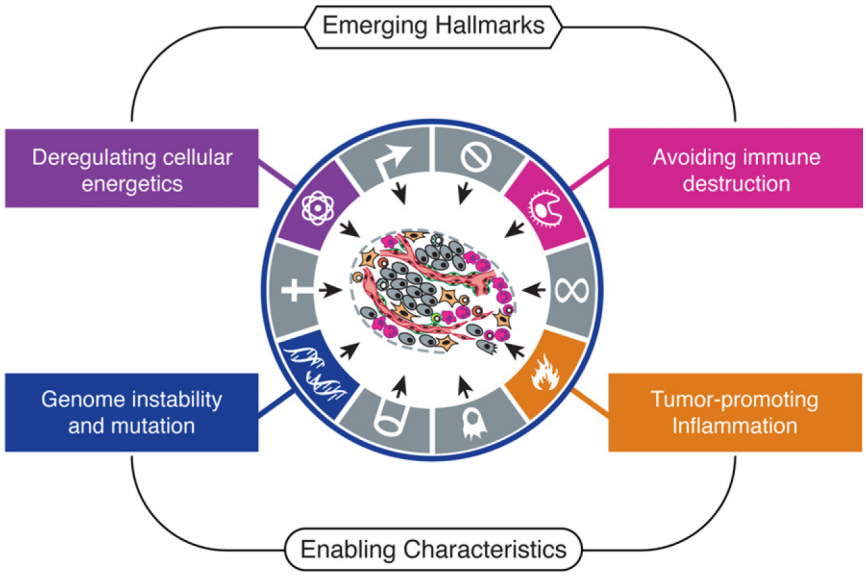



**Figure 1**. **Genomic Instability is an "enabling characteristic" of cancer.** In addition to the six established hallmarks of cancer four additional hallmarks of cancer have emerged. Two of them (including genome instability and mutation) are also considered enabling characteristics as they progressively promote neoplastic transformation (Adapted from Hanahan and Weinberg, 2011).

A very recent model classifies genomic Instability as one of the "enabling characteristic" among the hallmarks of cancer (Figure 1). Genomic instability could be initiated due to defects in genes of DNA maintenance and repair and once initiated, it provides further impetus to circumvent or abrogate other cellular barriers to carcinogenesis. For example, mutations in DNA double-strand break (DSB) repair genes breast cancer susceptibility 1 (BRCA1) and breast cancer susceptibility 2 (BRCA2) are frequently found in breast and ovarian cancer patients, whereas alterations in DNA mismatch repair proteins hMLH1, hMLH2 and hMSH2 are frequently observed in colorectal cancer patients (Dietlein et al., 2014). Prostate

cancer patients also exhibit frequent inactivating mutations in the DNA repair pathways (Leongamornlert et al., 2014).

Inherited defects in DDR genes also give rise to some rare hereditary diseases, marked by phenotypes such as neurodegeneration, infertility, immune deficiencies, growth retardation, premature aging and a marked predisposition to cancer. Many of these inherited diseases share common phenotypes. For example, Xeroderma pigmentosum (XP) was the first rare autosomal recessive syndrome identified with a defect in a DNA repair pathway. The cells from all XP patients show an increased frequency of mutations after exposure to UV light that is correlated with defects in repair of thymidine dimers. XP patients also develop progressive neurological problems (Menck and Munford, 2014). One of the most extensively studied neurodegenerative diseases associated with defect in DNA repair genes is ataxia telangiectasia (A-T), which is caused by mutations in the ataxia–telangiectasia mutated (ATM) gene. A-T patients exhibit profound ataxia, other neurological defects such as defective eye movement, speech defects, and non-neurological symptoms such as the absence or the rudimentary appearance of a thymus, immunodeficiency, insulin-resistant diabetes, radiosensitivity, cell cycle checkpoint defects, chromosomal instability and predisposition to cancer. A-T patients are confined to wheelchair in very early years of life and usually die of malignancies in their teens (Biton et al., 2008). As described before the role of DNA damage repair in the generation of gametes and the development of the immune system, defects in the genes of DNA repair often results in infertility and immunodeficiency.

The DDR protein most frequently mutated in cancers is p53. It is a transcription factor that stimulates transcription of the genes that promote cell cycle arrest, genomic integrity and apoptosis (Hanahan and Weinberg, 2011; Jackson and Bartek, 2009; Kastan, 2008). p53 mediates apoptosis mainly by stimulating the transcription of various proapoptotic genes, including members of the Bcl-2 family, such as Bax, Noxa, and Puma; although p53 is also implicated in the repression of anti-apoptotic genes, such as *survivin* (Kang et al., 2005; Zilfou and Lowe, 2009). Loss of p53 function, through mutations in p53 itself or alterations in pathways signaling to p53

is frequently observed in many human cancers. The Li-Fraumeni syndrome is caused by inheritance of mutations in the p53 gene and the afflicted individuals have a very high risk, almost 100% of developing many different cancers in their lifetime (Kastan, 2008).

## 1.2. Sources of DNA damage

### 1.2.1. Endogenous DNA damage sources

Genotoxic stress can either be endogenous or result from exogenous sources. Endogenous sources are mainly hydrolytic spontaneous reactions and metabolic by-products that leads to the generation of reactive oxygen species (ROS) and nitrogen species, lipid peroxidation products, endogenous alkylating agents, estrogen and cholesterol derived reactive species, and reactive carbonyl species. Of all the endogenous agents that attack DNA, ROS are the most predominant source of endogenous DNA damage. ROS include hydrogen peroxide, hydroxyl radicals, superoxide, and singlet oxygen. All these highly reactive molecules cause oxidative stress when the production of ROS exceeds the natural limit of the body to counterbalance the oxidative load. Under these conditions the three major biological macromolecules DNA, proteins and lipids are oxidized. In DNA ROS can lead to oxidized bases as well as single and double-strand breaks. Among the oxidized bases, 8-oxo-deoxyguanosine (8-oxo-dG) is the most abundant one. 8-oxo-dG base preferentially pairs with adenine rather than cytosine and thus generates GC to TA transversion after DNA replication, leading to point mutations (De Bont, 2004). The polyunsaturated fatty acid residues of phospholipids are also targeted by ROS, which leads to the production of intermediate products lipid hydroperoxides that are converted to unreactive fatty acid alcohols, epoxides and aldehydes. Malondialdehyde (MDA) is the most mutagenic product of lipid peroxidation. Direct oxidation of DNA by agents that extract the 4'-hydrogen atom of the sugar backbone leads to the formation of base propenals through a series of reactions. Base propenals are more potent than MDA in formation of exocyclic adducts (De Bont,

2004). DNA bases are also susceptible to hydrolytic deamination. Cytosine and its homologue 5-methylcytosine are the main targets of deamination (Lindahl, 1993). It was estimated that around 100 to 500 cytosines are deaminated to uracil in a single cell per day (Lindahl, 1993) and this could lead to GC to AT transitions, eventually.

### 1.2.2. Programmed DNA damage

Although most of the DNA damage is harmful to the cells, there are instances where a cell deliberately induces DNA lesions to induce genetic variations. Two examples of cells that undergo programmed induction of DNA double-strand breaks (DSBs) are lymphocytes and developing germ cells. Developing lymphocytes have evolved genetic mechanisms to enable the adaptive immune systems to generate a vast repertoire of immunoglobulin and T-cell receptor (TCR) diversity to deal with almost any kind of antigen. Unlike any other genes in metazoans, immunoglobulin (Ig) and TCR genes encoded in segments that must be recombined with flanking recombination signal sequence (RSS) during B- and T-cell development to generate complete genes. Two RAG proteins encoded by RAG1 and RAG2 genes introduce DSBs precisely between the gene segments to be joined and their flanking recombination signal sequences (Schlissel et al., 2006). RAG proteins then initiate rejoining of DSBs by recruiting enzymes involved in DSB repair in all cells and also by holding the ends of cleaved DNA in close proximity (Bassing and Alt, 2004). The cells switch generation of membrane-bound IgM and IgD to IgG, IgA, or IgE antibodies via a recombination based process, called as Class-switch recombination (CSR). CSR differs from V(D)J recombination in many ways, but the one that is most significant from DNA repair point of view is different mechanism for the induction programmed DSBs and differential requirement of components of the cellular DSB response for site specific recombination. For instance, CSR is highly dependent on p53-binding protein 1 (53BP1), while the role of 53BP1 in V(D)J recombination is rather limited (Schlissel et al., 2006).

Programmed DSBs are also crucial for the meiotic recombination step in the process of gametogenesis, known as meiosis. Meiosis produces gametes with half as many chromosomes as their diploid precursor cells. Meiotic recombination begins after the pairing of the homologous chromosomes, with the induction of programmed DSBs by a conserved protein called as Spo11. Spo11 breaks both strands of the DNA double helix in one of the homologous chromosomes via a topoisomerase-like reaction to generate transient, covalent protein-DNA intermediates at the 5' ends of the (Keeney, 2008; Neale et al., 2005). After DSBs are formed, Spo11 is removed from the 5' end of DNA in a two-step process. In the first step MRE11 endonuclease activity introduces a nick on the DNA duplex as far as 300 nt from DSB end (Garcia et al., 2012), which triggers Exo1-dependent 5'-3' resection and MRE11 exonuclease dependent 3'-5' degradation of the nicked strand toward the break. In the second step generation of 3' DNA tails via DSB resection, promotes recombination with the homologous chromosome. The recombination of meiotic DSBs with homologous chromosomes can either result in reciprocal exchange of the chromosome arms flanking the break (a crossover) or no exchange of flanking arms (a noncrossover). The reshuffling of genes by the process of meiotic recombination between maternal and paternal chromosomes increases the genetic diversity of the population.

### 1.2.3. Exogenous DNA damage sources

DNA is also damaged by an array of exogenous physical and chemical agents. Two notable examples of physical genotoxic agents are ionizing radiation (IR) and ultraviolet (UV). IR (from natural cosmic and earth radiation, medical treatments employing X-rays or radiotherapy) can induce oxidation of DNA bases and generates single-strand breaks (SSBs) and DSBs (Hoeijmakers, 2009). Some IR results from radioactive decay of naturally occurring radioactive compounds. For example an important source of IR is the natural occurrence of radioactive isotopes in the air, most notably Radon, which is generated by the decay of naturally occurring Uranium in the soil. The most pervasive environmental DNA-damaging agent is UV light. UV light has a wavelength ranging from 200 to 300 nm and is found in natural sunlight.

Although the ozone layer largely absorbs the most dangerous part of the UV light (UV-C), residual UV-A and UV-B in sunlight can induce $10^5$ DNA lesions per cell per day (Hoeijmakers, 2009; Lindahl and Barnes, 2000). UV radiation causes two classes of DNA lesions: cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PPs). Both CPDs and 6-4 PPs cause conformational perturbations in DNA in the form of primary lesions, mainly kinks or bends. Primary lesions can then be converted to secondary lesions when DNA replication or RNA transcription associated protein complexes hit primary lesions, leading to formation of DSBs.

Two major ways to treat cancer are chemotherapy and radiotherapy; both of these mostly depend on exogenous DNA damaging agents. DNA damage is normally recognized and repaired by the intrinsic DNA damage response (DDR) machinery. If the damaged lesions are successfully repaired, the cells will survive. An unsuccessful DNA repair might result in cell death. Cancer cells have evolved capabilities to ignore DNA damage and notably, they are also often incapable of temporarily halting cell-cycle progression in response to DNA damage. This means that they basically continue to divide in the presence of DNA damage. Given the fact that one of the hallmarks of cancer is high proliferation rates; treating cancer cells with DNA damaging agents often have lethal consequences, for example catastrophic levels of chromosomal loss and/or gross chromosomal rearrangements that occurs when a cancer cell divides despite the presence of chromosome breaks.

DNA crosslinking agents such as mitomycin C (MMC), cisplatin, psoralen, and cyclophosphamide introduce covalent links between bases of the same DNA strand (intrastrand crosslinks) or of opposite DNA strands (interstrand crosslinks or ICLs). ICLs represent the most deleterious lesions produced by chemotherapeutic agent as they block DNA strand separation, thus interrupting essential processes such as replication and transcription (Deans and West, 2011). ICL-inducing drugs including melphalan and cisplatin are among the most widely used chemotherapeutic agents. Chemical modifications of nitrogen mustards such as cyclophosphamide and melphalan are widely used as chemotherapeutic agents until now in the treatment of leukemia and solid tumors (Deans and West, 2011). Topoisomerases are a class of

enzymes responsible for releasing the torsional strain in front of the DNA replication and transcription machineries. A DNA topoisomerase can be viewed as a nuclease that breaks a phosphodiester bond, reversibly in a DNA strand by attaching covalently to the phosphate of the DNA backbone. Topoisomerase I allow the passage of a single DNA strand through a transient single-strand break created in the complementary strand of the double helix. Topoisomerase II cuts both strands of the double helix to allow the passage of an intact helix to unwind supercoiled DNA. Topoisomerase inhibitors are used to treat certain leukemias, as well as lung, ovarian, gastrointestinal, and other solid cancers. Topoisomerase inhibitors camptothecin (CPT) and etoposide (ETOP) inhibit topoisomerase I and II, respectively. Topoisomerase poisons trap the DNA-enzyme intermediate complex and this impedes replication fork progression resulting in the formation of DSBs (Nitiss, 2009). Another class of drugs that is widely used to treat cancer is anthracyclines. Doxorubicin and Epirubicin, are among the most frequently used anthracyclines. Anthracyclines contain an amino sugar linked by a O-glycosidic bond to the quinone group of a polyaromatic ring (Minotti, 2004). For many years now, mechanism of action of anthracyclines is under active investigation and it seems that in addition to acting as Topoisomerase II poisons, anthracyclines also act as intercalating agents, oxidizing agents, DNA alkylating agents and crosslinking agents. They also interfere with helicase activity of DNA helicases and induce apoptosis (Minotti, 2004). Alkylating agents such as methyl methanesulfonate (MMS) and temozolomide attach alkyl groups to DNA bases. Most reactive groups that can draw up covalent bonds with alkylating agents are present in guanine. These covalent modifications disturb replication and transcription, eventually resulting in cell death. Alkylating agents are more effective in treating slow-growing cancers (Ralhan and Kaur, 2007).

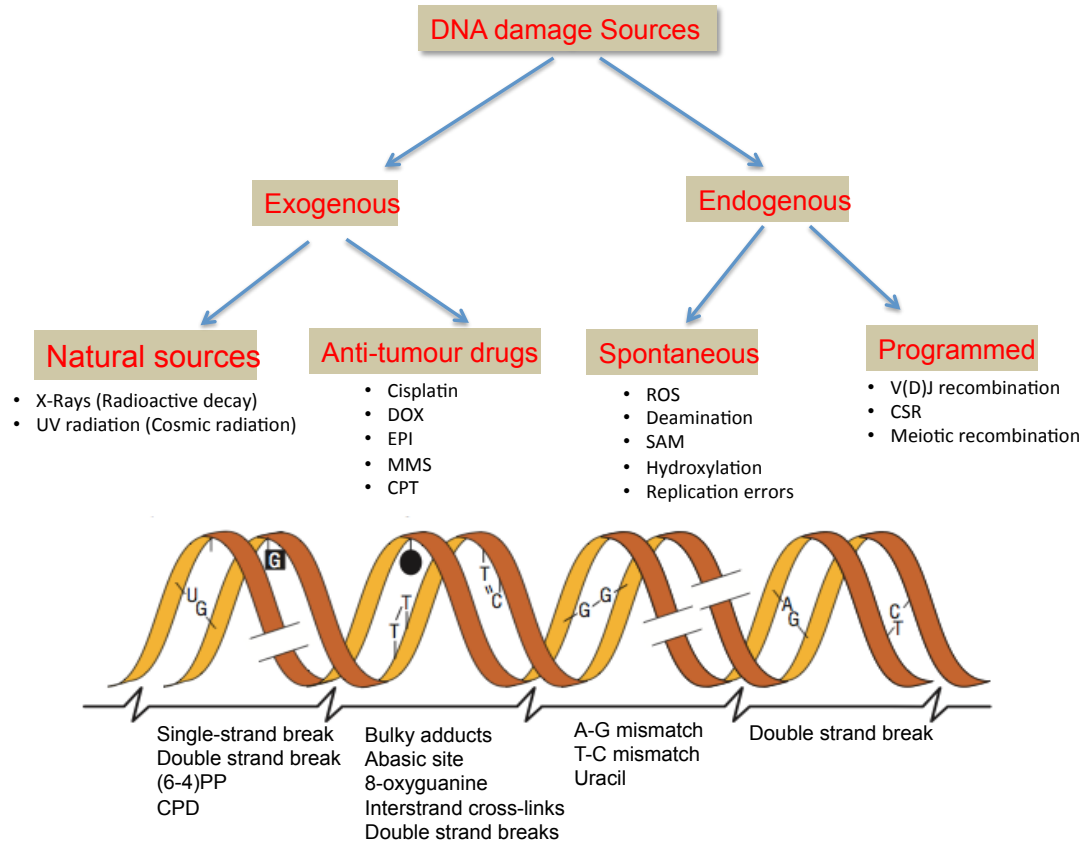



**Figure 2. Sources of DNA damage.** Natural sources such as X-Rays are the most common sources of exogenous DNA damage. UV light induces the formation of 6–4 photoproduct (6–4 PP) and cyclobutane pyrimidine dimer (CPD). Anti-cancer drugs like cisplatin (*cis*-Pt) and mitomycin C (MMC) are DNA-crosslinking agents that generate interstrand cross-links. Topoisomerase poisons such as camptothecin (CPT) irreversibly bind to DNA-topoisomerase complexes thus physically impede progression of replication forks, which often results in the formation of DSBs. Doxirubicin and Epirubicin belong to a group of drugs known as anthracyclines, which can act as intercalating agents, topoisomerase II poisons, oxidizing agents and crosslinking agents Endogenous sources are mainly hydrolytic spontaneous reactions and metabolic by-products that lead to the generation of reactive oxygen species (ROS) and nitrogen species, lipid peroxidation products and reactive carbonyl species, all of which damage DNA. Most of the programmed DNA damage in the cell leads to the formation of DSBs. Programmed DSB's are important for genetic diversity and immunological responses. Adapted and modified from (Hoeijmakers, 2001).

### 1.3. DNA damage response - A broad overview

The DNA damage response (DDR) is a collection of biological processes, which are highly conserved throughout evolution and exist both in prokaryotes and eukaryotes to counteract the deleterious effects of DNA damage. The importance of genome maintenance is evident from the large repertoire of proteins that has evolved to ensure that the information encoded by the DNA is not corrupted. These investments include the double-helical structure of DNA that provides a back-up of the information in case one strand of the double-helix is damaged, multiple DNA damage recognition, signaling and repair systems; programmed cell death if DNA damage is beyond repair and proofreading activities employed at multiple levels for faithful replication of the genome. The DDR is a biological signaling network by which cells have integrated DNA repair pathway choice and coordination of that pathway with transcription, cell cycle progression, replication and apoptosis**.** The DDR is controlled by three apical phosphatidylinositol 3-kinase-like protein kinase (PIKKs), ataxia–telangiectasia mutated (ATM), ataxia–telangiectasia and RAD3- related (ATR) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs).

ATM and DNA-PKcs activation is induced by DSBs. The basic structure that leads to ATM activation is believed to be free DNA ends (You et al., 2007). Multiple types of DNA lesions activate ATR, including DSBs, base adducts, crosslinks, and replication stress. Many studies have shown that the basic structure that activates the ATR response in the cell is long stretches of single-stranded DNA (ssDNA) (Cimprich and Cortez, 2008). Once activated, these kinases phosphorylate a multitude of downstream targets such as factors of the DNA repair apparatus, factors of the cell-cycle machinery and factors that regulate the apoptotic programs. They also turn on various transcriptional programs. Recruitment of the apical PIKKs to DNA lesions is important for their activation and occurs through direct interactions with specific adaptor proteins that act as DNA damage sensors. For example, Nijmegen breakage syndrome 1 (NBS1) is an adaptor of ATM, ATR interacting protein (ATRIP) is an adaptor of ATR and KU70/80 heterodimer acts as an adaptor for DNA-PKcs (Falck et al., 2005; You et al., 2005). In addition to DNA repair and checkpoint activation, ATM

and ATR coordinate a wide spectrum of cellular activities ranging from DNA replication to transcription, metabolic signaling, and RNA splicing (Ciccia and Elledge, 2010). Many effector pathways of the DDR are regulated by the kinases ATM and ATR via phosphorylation and activation of the two transducer kinases checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2). Downstream targets of Chk1 and Chk2 are known as effector proteins that function at the interface between the cell cycle, DNA repair and apoptosis. Either ATM/ATR or Chk1/Chk2 then targets different proteins alone or alternatively, ATM/ATR and Chk1/Chk2 target distinct residues of the same effector proteins, respectively. One such effector protein is the transcription factor p53, which is implicated in many aspects of the DDR including cell-cycle control, DNA repair, apoptosis, senescence, and organismal aging (Fei and El-Deiry, 2003).

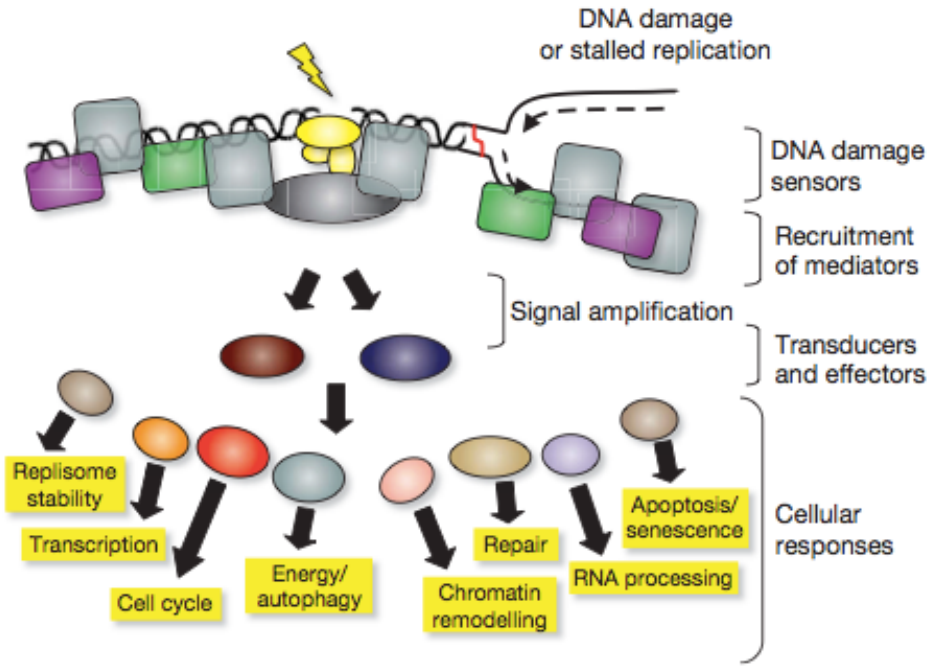



**Figure 3. DNA damage Response (DDR).** DNA lesions elicit signal transduction pathways that involve a set of proteins categorized as sensors, mediators, transducers and effectors. DNA damage is sensed by sensors, which then transmit the signal to checkpoint transducers which in turn transmit and amplify the DDR signal to downstream targets by influencing the activity or stability of DNA-repair factors, cell-cycle control factors, chromatin remodeling enzymes and apoptotic control proteins. Adapted from (Jackson and Bartek, 2009).

### 1.3.1. Cell cycle checkpoint activation

The cell cycle is an ordered sequence of events that ultimately leads to duplication of the genetic material. The eukaryotic cell cycle is divided into four sequential phases: G1 (first gap phase), S phase (DNA replication phase), G2 (second gap phase) and M phase (cell division). Cyclin dependent protein kinases (CDKs) are the major regulators of the cell cycle. CDKs are the catalytic subunits that dimerize with specific regulatory subunits to form Cyclin/CDK complexes. In humans, 11 genes encode different CDKs that associate with specific Cyclins in a cell cycle dependent manner (Malumbres and Barbacid, 2009). The Cyclin/CDK complexes are phosphorylated by a CDK-activating kinases (CAKs) to form active complexes (Morgan 1995). In order to elicit checkpoint activation, CDKs are inactivated either by the actions of kinases or CDK inhibitors (CKI) (Malumbres and Barbacid, 2009). To ensure that the structural integrity of the DNA is maintained, the cells have evolved different control mechanisms known as cell cycle checkpoints. The checkpoints have evolved to temporarily arrest cell cycle progression at different critical transitions of the cell cycle, upon the presence of genotoxic stress. The checkpoint activation can potentially be invoked at three phases of the cell cycle. The first checkpoint is in late G1 (G1S checkpoint), where either the cell commits to enter S-phase or draw itself out of the cell cycle by entering a quiescence state (G0). To prevent transmission of corrupted genetic information to the daughter cells, cells in G1 phase repair any damage that is generated by endogenous ROS species, chemical agents, UV or IR (Branzei and Foiani, 2008). Only when the DNA is free of any lesion and external factors like mitogens are adequate, the cell makes the transition to the S phase.

The next checkpoint is executed during S phase (intra-S phase checkpoint). The physical impediments to the elongating replication fork — such as DNA damage, DNA/protein adducts, non-B DNA structures and collisions with transcription machinery, makes S phase the most vulnerable period of the cell cycle (Ciccia and Elledge, 2010; Gaillard et al., 2015; Hoeijmakers, 2009; Zeman and Cimprich, 2014). In response to DNA damage during S phase, the cells transiently delay the progression of the cell cycle in order to allow time for the repair of the damaged

DNA. If damage is not repaired during this delay the cell exits S phase and arrests later when it reaches G2 checkpoint (G2 accumulation), which blocks cells from entering mitosis with damaged DNA. If there are problems in the completion of DNA replication because of DNA damage, the cell stays arrested in G2 until those problems are resolved. Replication associated checkpoints (Replication checkpoint) is different from the DSB induced checkpoint (intra-S phase checkpoint), but all the S-phase checkpoints share some common apparatus and are well integrated mutually. Intra-S-phase checkpoint defect is manifested in the form of inability of cells to transiently delay the cell cycle progression by inhibiting the initiation of replication origin firing and thereby slowing down DNA replication. The IR induced DNA damage in cells leads to the phosphorylation of CDC25A by Chk1, activated by ATR (Mailand et al., 2000; Negrini et al., 2010) and Chk2, activated by ATM (Dietlein et al., 2014; Falck et al., 2001). Phosphorylation of CDC25A triggers its ubiquitin dependent proteaosomal degradation; as a consequence CDC25A is no longer available to remove the inhibitory phosphorylation on CDK2 (Bartek and Lukas, 2001; Bartek et al., 2004). This eventually results in inhibition of CDK2-dependent loading of initiator of DNA replication CDC45, onto chromatin associated prereplicative complex (Costanzo et al., 2000). Another important checkpoint, that elicits in response to DNA damage is G2M checkpoint, which id described separately in the next section.

### 1.3.2. G2M checkpoint activation

The third important DNA damage induced cell cycle checkpoint is at the transition from G2 to M phase, before the cell cycle control system allows the cell cycle progression into mitosis. The cell has linked the demand for accurate repair of DNA lesions and the opportunity of having a sister chromatid by this time to the most accurate and faithful recombination based pathways of DNA repair (HR), that are elicited during the activation of G2M checkpoint. IR induces two temporarily and mechanistically different checkpoints in G2 (Figure 4). The first one is activated immediately after IR exposure and it stops the progression of cells that are in G2

phase to enter M phase. This checkpoint is ATM dependent and is generally known as G2M checkpoint. The other is known as G2 accumulation checkpoint. G2 accumulation checkpoint is ATR dependent and it is actually enhanced by the lack of ATM, as well as NBS1. The activation of this checkpoint is typically assessed several hours after IR. The activation of this checkpoint leads to the accumulation of the cells that had been in earlier phases of the cell cycle at the time of irradiation in G2. G2 accumulation is more pronounced in cells that are defective in IR-induced intra-S phase checkpoint, such as BRCA1 deficient cells (Xu et al., 2002).

Central to the regulation of the G2M checkpoint is the inhibition of the CDC25C phosphatase, a protein that removes the inhibitory phosphorylations from cyclin B/CDK1 kinase. CDC25C dependent dephosphorylation of cyclin B/CDK1 is vital for the activation of M-CDK at the beginning of mitosis. This happens via different ways; in one pathway activated Chk2 phosphorylates CDC25C at Ser216 to block its function. In a parallel pathway activated Chk1, negatively regulates CDC25C by phosphorylating it at Ser216 (Lammer et al., 1998; Matsuoka et al., 1998; Peng et al., 1997). Other upstream regulators of CDC25C and/or cyclin B/CDK1 that can also be targeted by DNA-damage-induced mechanisms are Polo-like kinases PLK3 and PLK1 (Ando et al., 2004). Interestingly, CDC25A and CDC25B have also been implicated in the activation of G2M checkpoint and it is now becoming more and more clear that CDC phosphatases play a more redundant role in the activation of cell cycle checkpoints (Ferguson et al., 2005; Iliakis et al., 2003; Kastan, 2008; Lammer et al., 1998; Melixetian et al., 2009; Niida, 2005). The G2M checkpoint also depends on the transcriptional programs initiated by BRCA1 and p53, leading to the upregulation of cell-cycle inhibitors such as p21 (Fei and El-Deiry, 2003; Kang et al., 2005; Zilfou and Lowe, 2009).

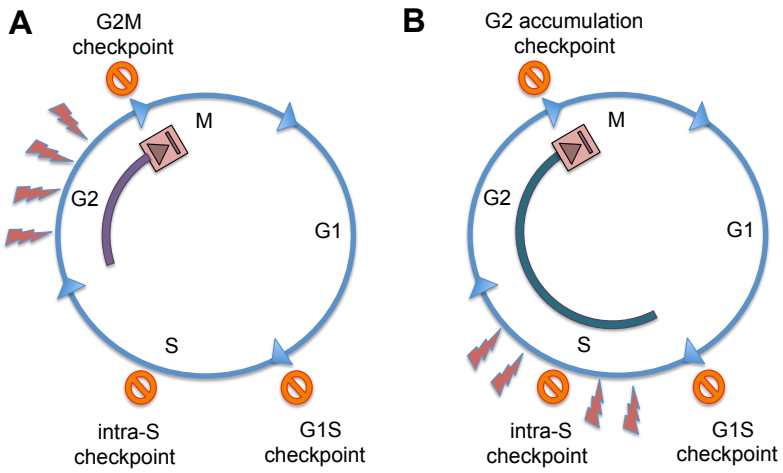



**Figure 4. Two molecularly distinct checkpoints are elicited at the G2 to M phase transition.** These checkpoints prevent the progression of cells into the M phase in the presence of unrepaired DNA damage. **(A)** G2M checkpoint is activated in the cells that are in the G2 phase at the time of irradiation. This checkpoint is ATM dependent and it is activated as soon as 30 minutes after irradiation. **(B)** G2M accumulation checkpoint is activated in the cells that are in S phase at the time of irradiation. This checkpoint is ATR dependent and its activation can be monitored at later time points after irradiation.

### 1.3.3. DNA repair mechanisms

Cells have evolved diverse sophisticated repair mechanisms to deal with different types of lesions. DNA repair pathways are highly conserved and most types of DNA repair depends on the presence of a separate copy of the genetic information in the undamaged strand of the DNA double helix. At least five main, partly overlapping damage repair pathways operate in mammals — nucleotide-excision repair (NER), base-excision repair (BER), mismatch repair (MMR), non-homologous end-joining (NHEJ), and homologous recombination (HR). More subtle changes to DNA like oxidized bases, alkylation bases and SSBs, are repaired by BER. Relatively bulkier base adducts that distort the DNA helical structure like pyrimidine dimers such as those caused by ultraviolet light, are processed by NER. The MMR repair pathway repairs post-replication mismatched bases, which are escaped by the proofreading activity of the DNA polymerases and incorporated during replication. In addition to mismatched bases, MMR proteins also correct small insertions/deletions generated at repetitive DNA sequences due to polymerase slippage during replication. DSBs generated either by endogenous or exogenous sources are repaired by NHEJ or HR.

## 1.4. Repair of DNA double strand breaks (DSB's)

DSBs are the most lethal kind of damage and research has shown that even a single DSB is sufficient to kill a cell (Bassing and Alt, 2004; De Bont, 2004; Lettier et al., 2006). The cell has evolved multiple pathways that repair DSBs in contexts of complexity of DSBs, their location with respect to DNA as well as nucleus and cell cycle phase. Various studies using different experimental systems has led to the conclusion that DSB repair is essentially categorized into two broadly distinct and complementary pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ), although there could be several variations in the precise mechanisms of NHEJ/HR (Chapman et al., 2012b). NHEJ is extremely fast, functions throughout the cell cycle, operates with high efficiency and can result in small sequence alterations and occasional translocations. Whereas NHEJ does not require extensive resection of the break, HR depends on the generation of extensive ssDNA tracts by DNA end resection. HR is the only accurate pathway of DNA DSB repair, but it operates with slow kinetics and it is restricted to S and G2 phases of the cell cycle. Both these two major pathways of DSB repair are described in detail later in this chapter. In addition, there are pathways of DSB repair machinery that have harnessed components both from HR and NHEJ. DNA end resection is an important initial step of these pathways that are dependent of microhomologies. In case of alternative NHEJ (alt-NHEJ), DNA end resection reveals short regions of microhomology (5-25 bp), while in single-strand annealing (SSA), longer stretches of microhomology are revealed (Chiruvella et al., 2013; Jasin and Rothstein, 2013).

### 1.4.1. Non-homologous end joining (NHEJ)

NHEJ is the predominant DSB repair pathway in higher eukaryotes and it is active throughout the cell- cycle. This well-characterized DSB repair pathway operates with high speed, with a half time of 10-30 min and it efficiently removes DSBs from the genome. Genetic defects in its core components are associated with increased radiosensitivity. NHEJ is a mechanism where the broken ends are ligated after

limited modification. DSB repair by NHEJ often results in nucleotide deletions and/or insertions at repair junctions, which makes it more liable to errors. DSB repair via NHEJ can operate via two pathways: c-NHEJ (Classical NHEJ) or alt-NHEJ (alternative, microhomology mediated end joining or MMEJ). C-NHEJ appears to be the predominant DSB repair pathway used in mammalian cells and is active throughout the cell cycle, prominently in G0/G1. In c-NHEJ broken DNA ends are recognized by the KU70 and KU80 heterodimer, which form a symmetrical ring by direct contact with the DNA backbone or bases (Deriano and Roth, 2013). Association of KU with DNA ends serves both as a scaffold for the assembly of the NHEJ apparatus and a physical barrier to DNA end resection (Decottignies, 2013). KU heterodimer recruits DNA-PKcs and by interacting with KU-bound DNA ends, the kinase activity of DNA-PKcs is stimulated. The complex of KU and DNA-PKcs at the broken DNA ends protects the DNA from nuclease attack and the KU component of the DNA-PK complex contributes to the recruitment at the break site of the ligase complex (DNA LigaseIV/ XRCC4/XLF4). Compatible DNA termini that possess a 5' phosphate and a 3' hydroxyl group can be directly ligated by the c-NHEJ factors described above. However, in order to repair more complex DNA ends, DNA-PKcs recruits and regulates the activity of ARTEMIS, a nuclease involved in the processing of the DNA ends. Endonucleolytic activity of ARTEMIS/DNA-PKcs can resect a wide range of DNA end configurations, which could lead to small deletions. DNA polymerases (such as Polμ or Polλ) fill small gaps by misincorporation of nucleotides at the broken DNA ends, which could lead to small additions, contributing to heterogeneity at the DSB repair site (Lieber, 2010). The net result of the action of DNA processing enzymes at the site of NHEJ repair is the generation of compatible ends, which are eventually sealed by the ligase complex (Figure 5).

Alt-NHEJ was discovered as a back up DSB repair mechanism in absence of c-NHEJ and it is less efficient than c-NHEJ. Alt-NHEJ is more error prone than c-NHEJ, as it can even facilitate the joining of unrelated DSBs, leading to extensive variations at DSB joining sites and even to chromosome translocations (Decottignies, 2013; Deriano and Roth) . In alt-NHEJ, Poly (ADP-ribose) polymerase 1 (PARP1) has been implicated in DSB end sensing and it competes with the KU heterodimer (c-NHEJ) for

free DNA ends. As with NHEJ, Alt-NHEJ is also proposed to be the back up pathway of HR (Deans and West, 2011; Deriano and Roth, 2013) and some of the protein factors implicated in alt-NHEJ are the key HR proteins like MRN complex (Dinkelmann et al., 2009; Xie et al., 2009) and CtBP interacting protein (CtIP) (Lee-Theilen et al., 2011; Zhang and Jasin, 2011).

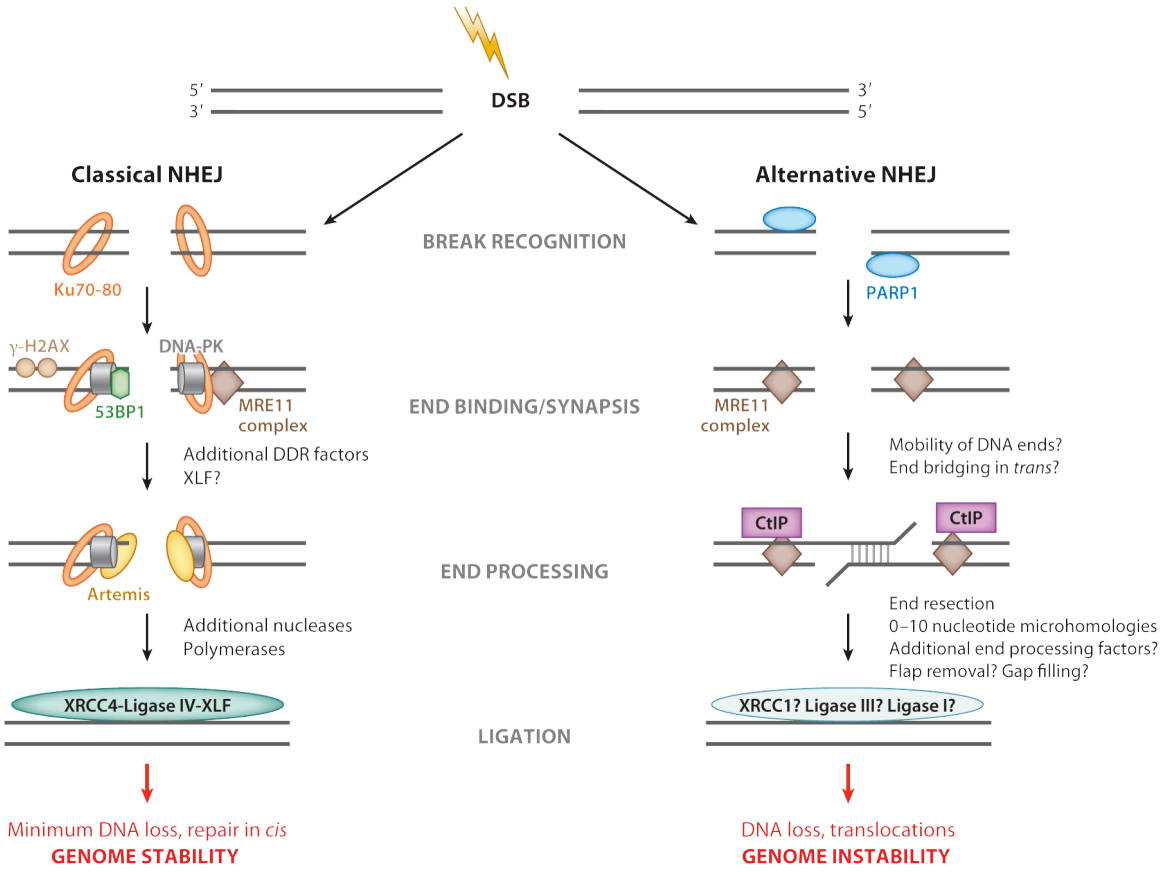



**Figure 5. Classical and alternative NHEJ pathways**. The DSBs are recognized and processed using different repair enzyme complexes in c-NHEJ and alt-NHEJ pathway. C-NHEJ is typically sensed by the KU70/80 complex, which then recruits DNA-PKcs. KU70/80 heterodimer and DNA-PKcs in conjunction with nucleases such as the MRN complex and ARTEMIS bring about the limited processing of the DNA broken ends, which are eventually ligated by a DNA ligase complex (XRCC4/Ligase IV and XLF). In alt-NHEJ broken ends are sensed by PARP1, which are then modified by the combined action of the MRN complex and CtIP. The clean ends generated are finally sealed by a ligase. Adapted from (Deriano and Roth, 2013).

### 1.4.2. Homologous recombination (HR)

HR is the most accurate pathway of DSB repair and it happens in the S and G2 phases of the cell cycle. The cell cycle dependent execution of HR is linked to the presence of an intact sister chromatid as a template for HR directed repair, which ensures that no essential sequence information is lost and that the repair does not lead to loss of heterozygosity. HR-mediated repair is initiated with the recognition and binding of DSB ends by the MRN complex and CtIP. MRN complex is recruited to the break ends and together with the cohesin complex SMC5/6, it helps in stabilizing the DNA ends and hold them together in close proximity (Feeney et al., 2010; Hopfner et al., 2002; Williams et al., 2007). MRN complex is also involved in recruitment and activation of ATM (Falck et al., 2005; Lee and Paull, 2005; Williams et al., 2007; You et al., 2005).

The repair of DSBs is mainly regulated at the level of HR via DNA end resection; a process that generates 3' single-strand overhangs. DNA end resection is a key determinant of DSB repair pathway choice, which commits cells to HR and is also required for activation of the ATR-mediated checkpoint response (Jazayeri et al., 2006). DNA end resection is a highly regulated process and once initiated, the DNA ends become poor substrates for binding by NHEJ proteins like KU and cells are committed to HR. Two key players that initiate DNA end resection are the MRN complex and CtIP. In *S.pombe* MRN recruits CtIP and together they collaborate to initiate resection, leading to the generation of 3' ssDNA overhangs that subsequently are coated by a single strand binding Replication protein A (RPA). All sub-pathways of HR share the same initial steps including processing the DSB to 3' overhanging tails, on which the RAD51 filament is assembled. RAD51 coated ssDNA presynaptic filament, mediates the homology search and DNA strand invasion. The loading of RAD51 onto ssDNA is a critical step in HR, as it generates a nucleoprotein filament that searches for homology by invading a nearby homologous duplex DNA template. BRCA2 facilitates the loading of RAD51 onto ssDNA by overcoming the inhibitory effect of RPA, while BRCA1 plays a modulatory role in the PALB2 and BRCA2 dependent loading of RAD51 repair at ssDNA tails (Liu et al., 2014). RAD54 is a bidirectional motor protein that uses ATP to translocate on dsDNA. RAD54 associates with and stabilizes the RAD51 presynaptic filament. The motor activity of RAD54

stimulates strand invasion into a homologous DNA template, which involves pairing with the complementary strand and displacement of the other strand, leading to the formation of a triple-stranded DNA structure also known as D-loop. RAD54 then directs the transition from DNA-strand invasion to DNA synthesis by dissociating RAD51 from heteroduplex DNA and by recruiting DNA polymerase to the invading 3' end of the D-loop (Heyer et al., 2006). To avoid lethal consequences of unrestrained HR activity cells have evolved anti-recombinases, typical examples are Srs2 (*S. cervisiae*) and PARI (vertebrates). Both of these anti-recombinogenic helicases have been shown to bind RAD51 and to disrupt RAD51-ssDNA filaments in vitro (Chapman et al., 2012b).

After DNA synthesis is primed by the invading strand repair can proceed via three main sub-pathways referred to as the canonical double strand break repair (DSBR), synthesis-dependent strand annealing (SDSA) and break-induced replication (BIR) (Figure 6). During SDSA the elongated newly synthesized part of the invading strand pairs again with the resected strand of the second DSB end, to form DNA joint molecules (JMs). RTEL1 promotes SDSA over DSBR to avoid crossover formation in mitotic and meiotic cells (Youds et al., 2010). RTEL1 is also implicated in the disassociation of JMs, at an early stage before initiation of repair synthesis by anti-recombinase helicases (Chapman et al., 2012b). Since this process only produces non-crossovers, SDSA is the preferred recombination-mediated DSB repair in post-mitotic cells in order to prevent loss of heterozygosity (LOH) or other genetic rearrangements. Alternatively, in DSBR pathway, the second DNA end is captured by the D-loop to form an intermediate with two Holliday junctions, referred to as double Holliday junction (dHJ). These joint DNA molecules can either be resolved by specialized endonucleases into crossover (CO) or non- crossover (NCO) products. Yen1/GEN1, SlLX1-SLX4 and MUS81-MMS4/EME1, all have been implicated in dHJ resolution that can yield both COs and NCOs, depending on the orientation of cleavage. However, nicked HJs (precursors of dHJ) might be the preferred DNA substrates of MUS81-MMS4/EME1 (Matos and West, 2014); which leads to the formation of COs (Chapman et al., 2012b). Another protein complex that exclusively leads to the formation of NCOs is BLM-TopoIIIα-RMI1-RMI2 complex (BTR). In mitotic

cells, BTR complex (STR in yeast) has been shown to migrate and decatenate dHJs, a process known as 'dissolution'. However in meiotic cells, MLH1, MLH3 and EXO1 have been implicated in resolution of dHJs, which primarily leads to the formation of COs (Matos and West, 2014).

Collapsed replication forks result in the formation of one-ended DSBs, which are predominantly repaired by break-induced replication (BIR) (Strumberg et al, 2000). The presence of RPA bound ssDNA adjacent to the stalled newly replicated double-stranded DNA and primer–template junction, act as a signal for the activation of the replication stress response (Cimprich and Cortez, 2008; Malkova and Ira, 2013; Zeman and Cimprich, 2014). The ATR pathway is activated in response to BIR and replication stress and leads to the inhibition of cell-cycle progression, suppression of late origin firing (global effects) and stabilization of stalled fork, restart of stalled fork, suppression of recombination (local effects). In this pathway, invasion of the 3'-stranded end into homologous duplex DNA generates a replication fork that replicates the template to the chromosome end. This process is potentially mutagenic because repair happens in a way that ensues LOH.

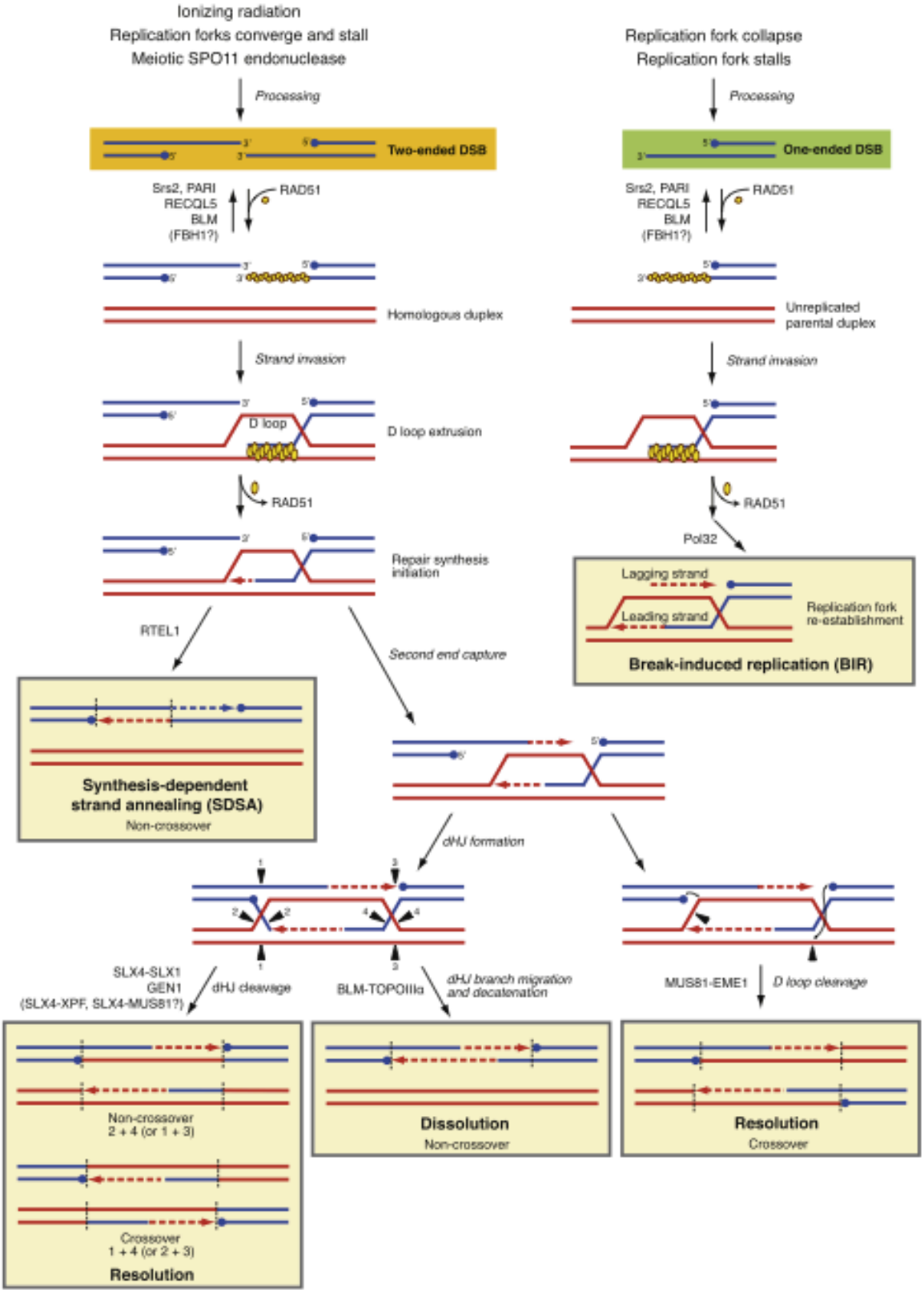



**Figure 6: Mechanism of Homologous recombination (HR).** Both two-ended and one-ended DSBs can be repaired by HR. Formation of 3' ssDNA tails by DNA end resection commits the cell to HR. In all the sub pathways of HR repair, RAD51 presynaptic filament invades the homologous duplex DNA and the broken DNA is extended followed either by second end capture to promote dHJ formation as in DSBR or disruption of D-loop as in SDSA. In BIR, Rad51 filament strand invasion reestablishes a replication

fork and the 3'-end is extended till the end of the chromosome template. Adapted from (Chapman et al., 2012b)

### 1.4.3. DNA end resection

DNA end resection is a crucial deterministic process for the fate of a DSB repair and it is believed to happen in two phases. In *S. cerevisiae*, this first step is dependent on the Mre11-Rad50-Xrs2 (MRX) complex (Symington, 2014). MRX/N complex and Sae2/CtIP are essential in all the pathways that require the processing of DNA ends like HR, alt-NHEJ and SSA (Bennardo et al. 2008; Simsek et al. 2011; Zhang and Jasin 2011). The precise role of MRX/N and Sae2/CtIP in DNA end resection is only beginning to be understood. It was unclear for a very long time as to how the MRN complex catalyzes 5'-3' nucleolytic degradation of DNA ends, as MRN exhibits 3'-5' exonuclease activity in vitro, which is opposite to the 5'-3' exonuclease activity required to produce 3' ssDNA overhangs. A general view now is that DNA-end resection occurs with bidirectional polarity, whereby the MRN complex incises DNA ends endonucleolytically and then uses its 3'-5' exonuclease activity to generate 3' DNA tails (Garcia et al., 2012; Shibata et al., 2013). It has been shown both in yeast and humans that Sae2/CtIP promotes nuclease activity of the MRX/MRN complex *in vitro* (Cannavo and Cejka, 2015; Sartori et al., 2007). Interestingly, the endonuclease activities have also been reported both for budding yeast Sae2 (Lengsfeld et al., 2007) and its human counterpart CtIP (Makharashvili et al., 2014). Although its still not clear if Sae2/CtIP acts as a regulator of MRE11 nuclease activity or as a nuclease itself, nevertheless the concerted actions of MRX/N and Sae2/CtIP are important to initiate resection.

The endonucleolytic incision of the 5' strand mediated by the actions of MRX/N and Sae2/CtIP leads to formation of short 3' ssDNA overhangs. Although this endonucleolytic cleavage step is not absolutely required to resect DSBs with "clean" ends, it is essential for the resection of DSBs with "dirty" ends, which are either blocked by covalently linked proteins or chemical adducts. Notably, the DSBs generated by IR have also "dirty" ends, due to oxidative damage of the DNA ends

(Nickjoo et al., 2001). In yeast, processing of clean and dirty ends is mediated via two separate pathways dependent on either the Sgs1/Dna2 (helicase/nuclease complex) or exonuclease 1 (Exo1) (Figure 7). Many studies have shown that mutants lacking Sae2 or the MRE11 nuclease activity were impaired only partially in DNA end resection, when the DSBs were generated by endonucleases, which generate “clean” ends (Clerici et al., 2005; Llorente and Symington, 2004). However, the DSBs, which have “blocked/dirty ends” such as those generated by IR treatment, methylating agents, camptothecin, etoposide or meiotic DSBs require MRX/N and Sae2/CtIP activity to initiate DNA end resection (Hartsuiker et al., 2009a; 2009b). All these observations have lead to the model, which proposes that MRX/N complex is not required for processing of “clean” DSBs, but it is absolutely essential for resection of non-canonical DNA ends (Figure 6).

MRX/N and Sae2/CtIP mediated endonucleolytic incision, create entry site sgs1–DNA2 and/or EXO1 (Huertas, 2010), but how these exonucleases carry out long-range resection in the 5’-3’ direction remained unclear for a very long time. The MRN/X complex and CtIP/Sae2 are required both for EXO1 dependent resection (Eid et al., 2010; Nicolette et al., 2010; Shim et al., 2010) and DNA2-BLM/Sgs1 complex dependent resection (Nimonkar et al., 2011; Shim et al., 2010). However, it has also been shown in yeast that Exo1 and Sgs1-Dna2 can resect clean DNA ends in an MRX independent manner (Cejka et al., 2010; Nicolette et al., 2010; Zhu et al., 2008), highlighting that MRN/X complex (first step of resection) is important for the repair of dirty or blocked ends. Major resection defects were only revealed when both Exo1 and Sgs1 were inactivated simultaneously, which indicates that EXO1 and DNA2-BLM/Sgs1 pathways play redundant roles during DNA-end resection (Gravel et al., 2008; Mimitou and Symington, 2008; Nimonkar et al., 2011). In contrast to yeast, MRN and CtIP are absolutely essential for DNA end resection, irrespective of the nature of the breaks (Cejka, 2015; Sartori et al., 2007).

Recent studies have indicated that processing of “blocked ends” is a bidirectional process (Figure 6). In the first step, concerted efforts of Mre11 and Sae2 initiate a nick in DNA strand up to 300 nucleotides upstream of 5'- terminus of the DSB. In the second step, Mre11 exonuclease activity provides the nicks in the 3’–5’direction

towards the DSB end and the Exo1 or Dna2-Sgs1 exonuclease activity in the opposing 5'-3' direction generate long stretches of 3' ssDNA tails/overhangs consisting of more than 50,000 nucleotides (Zhu et al., 2008).

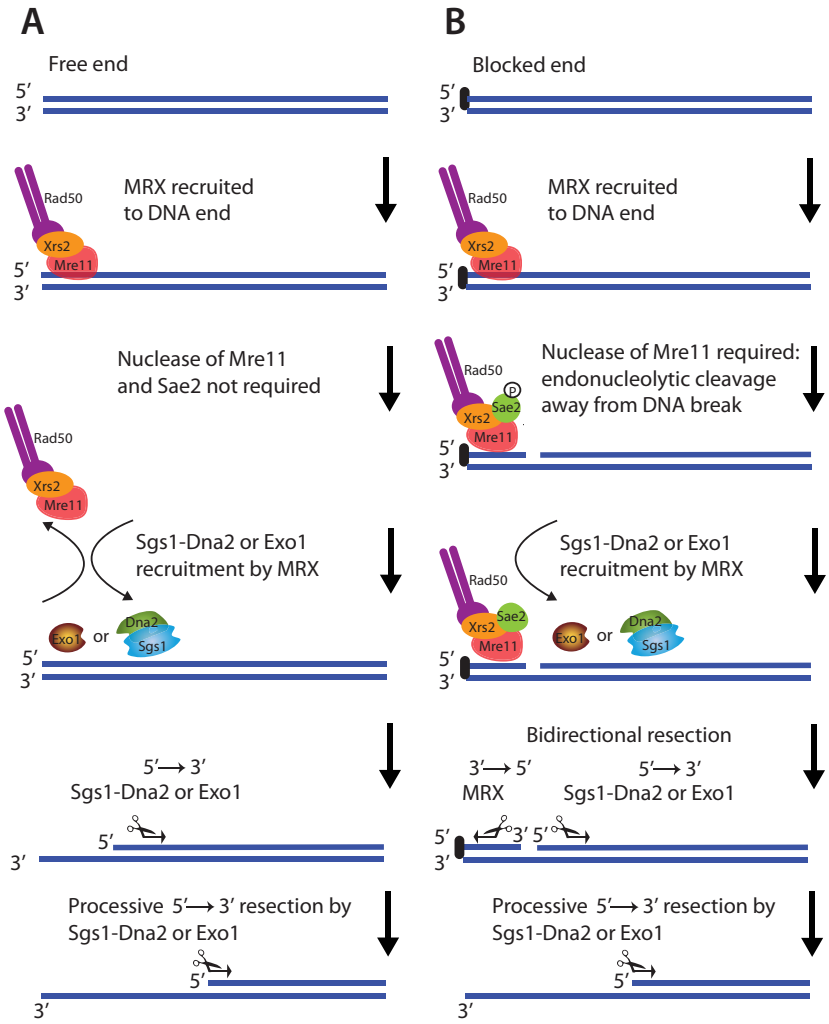



**FIGURE 7. DNA end resection of free and blocked DNA ends. (A)** The nuclease activity of Mre11 is not required for resection of free/clean DSBs. MRX complex is mainly required to recruit components of the long resection that include either Sgs1-Dna2 or Exo1. However, the role of the MRX complex in recruitment of Sgs1-Dna2 or Exo1 is not absolutely essential **(B)** In case of blocked ends, MRN/Sae2 complex is essential to initiate an endonucleolytic cut and to recruit Sgs1-Dna2 or Exo1, which then perform long range resection.

### 1.4.4. DNA end resection - determinant of DNA DSBR pathway choice

The default pathway of DSB repair in cells is NHEJ and HR is favored in S and G2 phases of the cell cycle when a sister chromatid is available as a repair template (Brandsma and Gent, 2012; Rothkamm et al., 2003). The default preference of the cell towards NHEJ is also evident from the fact that the Ku heterodimer can bind to the DSB much faster than HR factors (Kim, 2005). Even during S and G2 phases of cell cycle, NHEJ is the prominent DSB repair pathway and HR is only involved in the repair of few breaks. The choice between different DSBs repair pathways is thus highly regulated and DNA end resection is a crucial process in DSB repair pathway choice (Figure 8). DNA end resection is a critical regulatory step of HR. Two key NHEJ proteins KU and 53BP1 that subvert HR act primarily by blocking DNA end resection. KU binds DNA ends; it presumably physically blocks access of the DNA end resection machinery (Brandsma and Gent, 2012; Heyer et al., 2010; Jasin and Rothstein, 2013; Kass and Jasin, 2010; Mimitou and Symington, 2010; Pierce et al., 2001). DNA end resection machinery competes with KU and channels the repair of DSBs to HR in the S and G2 phases of the cell cycle. Not only HR, but also other pathways of DSB repair that involve resection like alt-NHEJ and SSA, are suppressed by KU (Weinstock et al. 2007; Simsek and Jasin 2010; Simsek et al. 2011; Escribano-Diaz et al. 2013).

53BP1 is another factor that protects DSB ends from processing by the DNA end-resection machinery during the G1 phase of the cell cycle. Stable binding of 53BP1 to DNA ends channels DSB repair into NHEJ in part by suppressing end resection. It has also been proposed that BRCA1 and CtIP collaborate to exclude 53BP1 and its cofactors, RIF1 and PTIP from the chromatin that is present around DSBs specifically in S and G2. Presence of 53BP1 and its cofactor RIF1 in the chromatin around DSBs in G1, blocks DNA end resection, while exclusion of 53BP1 from chromatin surrounding DSBs, lead to extensive end resection in S and G2 phases (Bouwman et al., 2010; Bunting et al., 2010; Callen et al., 2013; Chapman et al., 2012a; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013). Such a cell cycle poised regulation of DNA end resection ensures that HR is maximally activated only when sister chromatids are available to guide faithful repair. The importance of this cell cycle restricted balance between HR and NHEJ is evident in the absence of functional BRCA1, when DNA

lesions occurring in S-phase that are normally repaired through HR are instead channeled into NHEJ. This results in the formation of highly aberrant DNA repair products such as chromosomal radials, leading to lethal consequences to the cell (Chapman et al., 2012a).

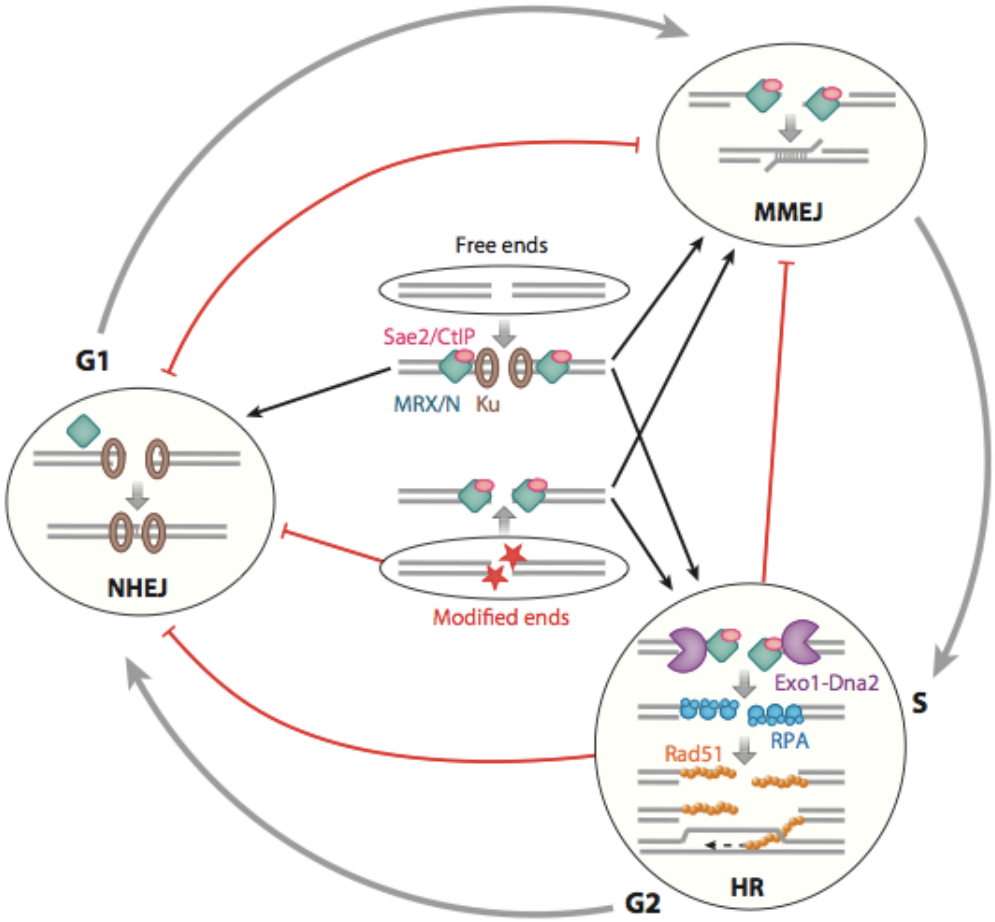



**Figure 8. DNA end resection as a regulator of DNA repairs pathway choice.** In G1 phase, NHEJ physically blocks DNA end processing via KU and prevents MMEJ or HR. In contrast, in S and G2 phases of the cell cycle MRX/N and Sae2/CtIP collaborate to initiate DNA end resection, which displaces KU from DNA ends and thus inhibits NHEJ. In case of HR there is extensive resection that results in RAD51 filament formation and inhibition of MMEJ. MMEJ is active throughout the cell cycle. Chemically modified or damaged ends (stars) cannot be repaired by NHEJ and require DNA end resection to be processed by MMEJ or HR. Adapted from (Symington & Gautier, 2011).

Apart from the tussle between the factors of HR and NHEJ, another important level of control that ensures that HR is maximally activated during the S and G2 phases of the cell cycle is exerted by CDKs. The CDK mediated phosphorylation of S/T-P motif at the Sae2-S267/CtIP-T847 is essential for DNA end resection (Huertas and Jackson, 2009; Huertas et al., 2008). MRN mediated phosphorylation of CtIP by CDK2, interferes with its proteaosomal degradation in S and G2 phases of the cell cycle (Buis et al., 2012; Ira et al., 2004). A recent study showed that phosphorylation-specific prolyl isomerase PIN1, upon phosphorylation by CDK by a proline-directed kinase PIN1 and CDK2, binds to CtIP and triggers its conformational change that results in ubiquitination and subsequent proteasomal degradation of CtIP (Steger et al., 2013). The degradation of CtIP is important, as the persistence of CtIP could lead to extensive DNA-end resection (Bonetti et al., 2015; Chen et al., 2015; Lafranchi et al., 2014; Nicolette et al., 2010; Puddu et al., 2015; Steger et al., 2013). CDK mediated phosphorylation of CtIP in S/G2 promotes its interaction with the other proteins of HR like MRN complex and BRCA1 (Chen et al., 2008; Yu and Chen, 2004). CDKs promote resection, both in yeast and vertebrates, and inhibition of CDK activity in G2 prevents extensive resection, Rad51 filament formation and Mec1/ATR activation (Ira et al., 2004; Jazayeri et al., 2006). DNA end resection is regulated at various levels, and in addition to cell cycle phase as described above, its regulation is also linked to chromatin structure and damage complexity (Clouaire and Legube, 2015; Symington, 2014).

### 1.4.5. Impact of DNA end resection on DNA damage signaling

DNA end resection generates long 3’ RPA coated ssDNA tails to which ATR/ATRIP binds and is subsequently activated by Rad17 and TopBP1 around ssDNA/dsDNA junctions. A critical target of ATR is the Chk1 kinase, which is required both for replication stress response and DNA damage induced checkpoint activation (Liu et al., 2006; MacDougall et al., 2007; Van et al., 2010; Zou and Elledge, 2003; 2003). Chk1 activity is regulated by its phosphorylation on S317 and S345 by ATR (Cimprich and Cortez, 2008). A recent study showed that Chk1 is activated rapidly after DSB

induction, in a manner independent of DNA end resection. This study also showed that DNA end resection is crucial for checkpoint maintenance, suggesting that resection is linked to prolonged cell cycle arrest to allow sufficient time for HR mediated DNA repair. The initial activation of the G2M checkpoint happens at a time point that precedes the initiation of DNA end resection. Once HR repair is initiated by DNA end resection, the resection dependent checkpoint maintenance pathway takes over and restricts mitotic entry until DNA is repaired completely (Kousholt et al., 2012). Thus, DNA end resection plays an important role not only in determining the DNA repair pathway but also in checkpoint maintenance, undermining the role of DNA end resection in eliciting an efficient overall DNA damage response.

### 1.5. The MRE11-RAD50-NBS1 (MRN) complex

The MRN complex is an important player in the DDR that is involved both in DSB repair and in the activation of DNA damage-induced signaling cascades. (Huertas, 2010; van der Linden et al., 2009). The MR complex is a heterodimer complex consisting of the two catalytic subunits, each of MRE11 and RAD50. This core complex is conserved from bacteria to archaea to eukaryotes and it is even found in some viruses like the bacteriophage T4 (Herdendorf et al., 2011; Hopfner et al., 2000b). The importance of MRN complex is reflected from the fact that the null mutations in any of the three components of MRN complex leads to embryonic lethality in mice (Williams et al., 2007). Hypomorphic mutations in any of the three components of MRN complex, lead to rare genetic diseases. Mutations in MRE11 gene lead to ataxia-telangiectasia-like disorder (ATLD). These patients have very similar phenotypes as ATM patients like ataxia and neurodegeneration (Taylor et al., 2004). Hypomorphic mutations in NBS1 in humans cause Nijmegen breakage syndrome (NBS), a rare autosomal recessive disorder characterized by microcephaly, immunodeficiency and cancer predisposition (Maser et al., 2001; Varon et al., 1998). It has also been reported that hypomorphic mutations in RAD50 lead to NBS-like disorder (NBSLD) (Waltes et al., 2009).

In eukaryotic cells, the MRN complex is comprised of a $MRE11_2RAD50_2$ ($M_2R_2$) heterodimeric core that further associates with NBS1, via interactions with MRE11 to give an overall stoichiometry of $MRE11_2RAD50_2NBS_2$; although there is some dispute over the number of NBS1 protein subunits bound to each $M_2R_2$ heterotetramer (Trujillo et al., 1998; van der Linden et al., 2009). The unusual structural architecture of the MRN complex is mainly characterized by RAD50 that features an extended rod-like shape (Figure 9A). The N-terminal and a C-terminal domain of RAD50 consists of walker A and Walker B domains that together form a bipartite ATP-binding cassette - ATPase (ABC-ATPase) (Hopfner et al., 2001; 2000a). A large helical region, which links the N- and C-terminal Walker domains of RAD50, folds into a long coiled coil tail that protrudes from the catalytic head region (Hopfner et al., 2002; Moreno-Herrero et al., 2005). The central part of RAD50 contains a highly conserved Cys-X-X-Cys sequence in the center of the coiled-coil region. This sequence between two RAD50 molecules can coordinate a zinc ion, to form a Zn-hook domain that leads to the formation an MRN intermolecular complex structure (Hopfner et al., 2002). RAD50 also contains MRE11 binding sites, located at the intersection of the coiled-coil and ATPase domains (de Jager et al., 2001; Hopfner et al., 2001). MRE11 further interacts with NBS1, via the MRE11 interaction sites present towards the C-terminus of NBS1 (Mirzoeva and Petrini, 2001; Petrini, 2003; Varon et al., 1998). The MRN complex thus consists of a globular head region, which mainly harbors the catalytic functions of the complex. It contains the MRE11 nuclease, the Walker A/B ATPase domain of RAD50. The other part is a large helical region that harbors a coiled–coil region and an apical zinc-hook dimerization motif that allows the interaction with other MRN complex (Figure 9B).

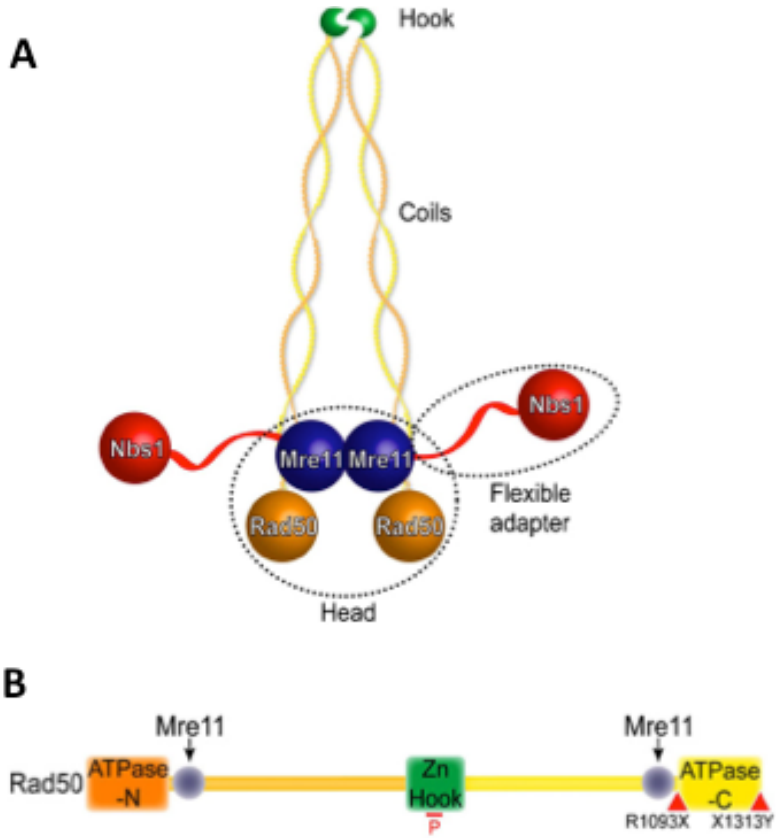



**Figure9. (A) Structural architecture of the MRN complex.** The head domain of the MRN complex consists of the ATPase domains of RAD50, together with MRE11 molecules. The coiled coil domain consists of the central regions of RAD50 and the Zn hook domain. The Zn hook domain connects two MRN complexes. NBS1 is connected via its C-terminal to MRE11. **(B)** The structure of RAD50. RAD50 contains Walker A and B motifs in N- and C-terminus, respectively, that are responsible for the ATPase activity of RAD50. The central part of RAD50 consists of two coiled-coil regions that are linked by a zinc- hook motif in the middle. MRE11 interacts with RAD50 via two regions localized at the intersection of the coiled-coil and ATPase domains. Adapted from (Williams et al., 2010).

### 1.6. NBS1: structure, function and link to diseases

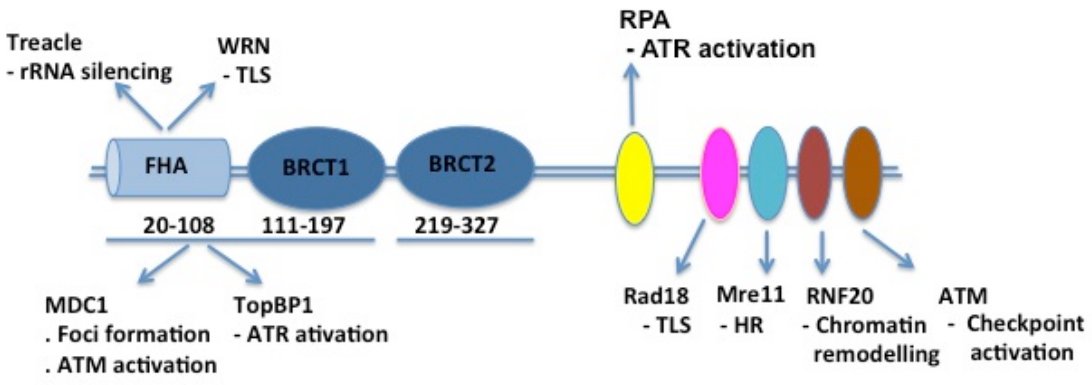
NBS1 is the least conserved component of the MRN complex and it is present only in eukaryotes. Although the NBS1 gene is widely conserved in function in higher eukaryotes, it shows a weak homology to the *S. cerevisiae* gene encoding Xrs2 at both the N- terminus and C-terminus and moderate homology to the *S. pombe* gene encoding NBS1 (Symington and Gautier, 2011). NBS1 is a regulatory subunit of MRN component and it mediates nuclear localization of MRE11 and RAD50 (Mirzoeva and

Petrini, 2001). Mutations in the MRE11 interaction motifs of NBS1 results in the cytoplasmic retention and degradation of MR (Desai-Mehta et al., 2001; Tauchi et al., 2001). NBS1 contains several functional domains, mainly in the N-terminus and the C-terminus. The N-terminus region of NBS1 includes two phospho-interaction domains, of which one is a forkhead-associated (FHA) domain and the other is the combined tandem 2 BRCA1 C-terminus (BRCT) domain. The C-terminal region of NBS1 harbors a MRE11 interaction motif, which is essential for the for direct binding to MRN complex and its nuclear transportation (Tauchi et al., 2001). Additionally, an ATM interaction site is also present at the very C-terminus of NBS1, which is essential for the recruitment of ATM to DSB sites and its activation (Difilippantonio et al., 2005; Falck et al., 2005; Lee and Paull, 2005; You et al., 2005., Shiotani et al., 2014). NBS1 mediated ATM phosphorylation events have been implicated in checkpoint activation (Buscemi et al., 2001; Girard et al., 2002).

The presence of FHA and BRCT domains, endows adaptor/mediator function to NBS1 (Figure 10). NBS1 does not possess any enzymatic activity and act as a adaptor for MR by mediating the sustained interaction of of MR with other proteins such as MDC1 in the DSB-flanking chromatin (Chapman & Jackson, 2008; Spycher et al., (Melander et al., 2008). In fission yeast, Nbs1 interacts with Ctp1 and this interaction is critical for promoting DNA damage resistance (Lloyd et al., 2009; Williams et al., 2009). In contrast, the physical and functional interaction between NBS1 and CtIP in human cells is much less clear. NBS1 is important in mediating phosphorylation of nuclear proteins by ATM (Buscemi et al., 2001; Gatei et al., 2003; Girard et al., 2002; 2002) and ATR (Stiff et al., 2005). NBS1 has also been implicated in the activation of ATR during replication stress (Duursma et al., 2013; Lee and Dunphy, 2013; Shiotani et al., 2014).

NBS1 belongs to the haploinsufficient tumor suppressor genes cohort, which includes several genes including many genes involved in DNA repair (Demuth and Digweed, 2007; Santarosa and Ashworth, 2004). The hypomorphic mutations in NBS1 gene lead to a rare autosomal recessive genetic disorder called as Nijmegen breakage syndrome (NBS). The most common mutation in NBS1 syndrome found in 90% of the patients (Varon et al., 1998) is deletion of 5 nucleotides in exon 6 of NSB1

(*657del5*). This mutation determines the synthesis of two proteins fragments p26 (26 kDa) and p70 (70 kDa). The p26 fragment includes 1-218 aa of the NBS protein, consisting of FHA and the BRCT1 domains. The p70 includes the second BRCT domain and Mre11 and ATM interaction sites (Krüger et al., 2007; Maser et al., 2001). NBS patients with defective NBS1 share many phenotypical features with AT patients, defective in ATM and ATR-Seckel patients, defective in ATR. For example NBS patients exhibit checkpoint activation defects, a feature shared by both AT and ATR-Seckel patients. However, immunodeficiency, chromosomal aberrations, genomic instability, cancer predisposition, heightened radiosensitivity, and developmental defects are observed only in AT and NBS patients. Whereas, microcephaly, mental retardation, dysmorphic facial features are observed only in ATR-Seckel and NBS patients. The risk of developing cancer in NBS patients is very high, with 50% of patients suffering for NBS developing lymphomas soon after the first decade of life (Demuth and Digweed, 2007). Increased cases of stomach and colorectal cancer have also been reported in NBS patients (di Masi and Antoccia, 2008). In comparison to decreased levels of NBS1 being implicated in most of the cancers observed in NBS1 patients, there are incidents where increased expression of NBS1 is linked to the development of several types of cancer, such as non-small lung cell cancer and uveal melanoma (Ehlers and Harbour, 2005). Increased NBS1 expression has also been suggested as a Marker of aggressive form of Head and Neck Cancer and Overexpression (Yang et al., 2006).




hNBS1 (754 a.a)

**Figure 10. Structure and major interaction partners of human NBS1.** FHA domain at the very N-terminal interacts with Treacle and WRN. Both FHA and BRCT domains of NBS1 interact with MDC1 and TopBP1. Several interaction domains at the C-terminus of NBS1 C-terminal are critical for binding to MRE11, ATM, Rad18, and RNF20. Adapted from (Saito et al., 2013).

### 1.6.1. FHA domains

The FHA domain was first discovered in forkhead family transcription factors and since then, it has been identified in more than 2000 proteins both in prokaryotes and eukaryotes (Mahajan et al., 2008). FHA domains can be found in many regulatory proteins, kinases, phosphatases and transcription factors, kinesin-like motors and regulators of small G proteins and have been implicated in diverse biological processes such as cell cycle control, cell growth, signal transduction and transcription and in DNA damage signaling. NMR or X-Ray crystallography has elucidated the structures of many FHA domains and some notable DDR examples include MDC1, NBS1, RNF8 and Chk2. The FHA domain spans approximately 80-120 amino acids, which form around 11 β-strands. These β-strands form two large intertwined anti-parallel β-sheets, which fold into a β-sandwich structure. Although the known FHA domains share low sequence homology but still adopt a similar fold, a structural featured attributed to the preservation of hydrophobicity in the β strands. The FHA domain specifically recognizes phosphothreonine residue and not phosphoserine residue. Combinatorial peptide library screening was used to identify ligand specificities for the different FHA domains (Durocher et al, 2000) and these studies elucidated that the highly conserved pThr and pThr+3 residue form the primary and secondary recognition site for binding to FHA domains. The pThr+3 residue could either be an Asp or Ile/Leu. However, there are exceptions to this pattern, e.g. the RNF8 FHA domain has a preference for Tyr or Phe at the pThr+3 position (Durocher et al, 2000; Huen et al, 2007) and the FHA domain found in yeast Dun1 has a unique preference for phosphorylated substrates including two consecutive pThr residues. The specificity of binding to phosphopeptides is determined by the loops and turns that connect the β-strands, which vary in length and α-helical insertions between the

different FHA domains. In addition to the role of FHA domains in kinase activation, they have been implicated in DNA damage dependent oligomerization processes as well. A well studied example is the FHA domain mediated transient dimerization of Chk2 kinase, that leads to its activation (Ahn, 2002). However, Chk2 dimer is believed to be a transient structural modification that places the kinase active sites in a way that facilitates their trans-phosphorylation, ultimately leading to their activation and then dissociation to active monomers. Lately, it has been shown that the FHA domain of MDC1 mediates an intermolecular interaction with a previously uncharacterized ATM phosphorylation site (pThr4) at the very N-terminus of MDC1, which leads to the dimerization of MDC1 in response to DNA damage

Like the characteristic FHA domain structure, the N-terminal 114 residues of NBS1 form 11 β-strands, which fold into a β-sandwich. As speculated from the sequence, analysis of the X-ray structure of the NBS1 N-terminal fragment revealed two tandem BRCT domains. The FHA domain and first BRCT domain exist as a single globular structure, contrary to what would be expected from two distinct domains. The FHA-BRCT1 domains form a compact structural fold unit that is linked to the second BRCT-repeat motif in a flexible way. The FHA/ BRCT2 domain composition is conserved in all eukaryotic NBS1 proteins. As mentioned before, the most common mutation found in NBS patients is *657del5* yields two protein fragments termed p26 and p70. (Maser et al., 2001) (Figure 9B). The underlying reason for this molecular scission is believed to be the instability of NBS1 because of exposure of a large hydrophobic patch which is normally buried in the BRCT1-BRCT2 Interface. The mutations in FHA domains can lead to tumorigenesis, as seen In the case of Chk2. Two missense mutations (Ile157Thr and Arg145Trp) in the FHA domain of Chk2 disrupt phosphorylation dependent protein-protein interactions and have been implicated in a variant form of the Li-Fraumeni cancer syndrome.

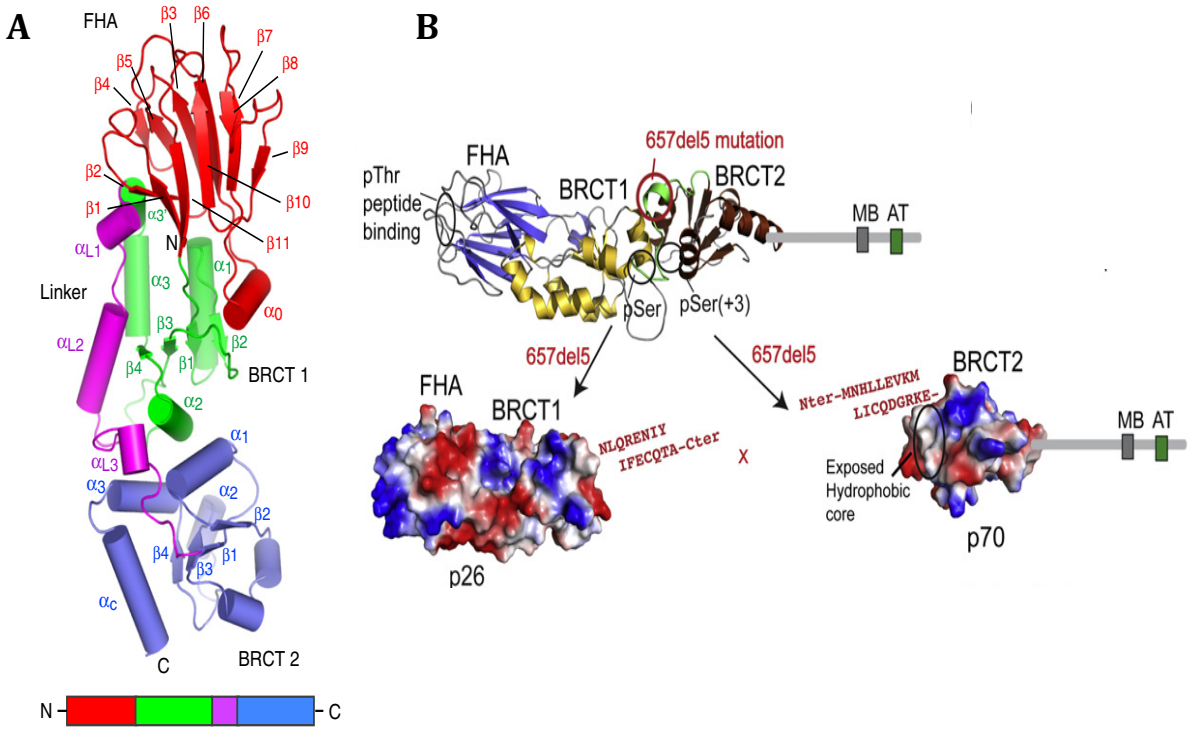



**Figure 9**: (A) Structure of FHA/BRCT domains of *S. pombe*. (B) NBS1 657del5 frameshift mutation found in majority of NBS patients causes a molecular scission of the protein that generates two protein fragments p26 and p70. The fragmentation of the protein leads to exposure of large patch of hydrophobic core, which leads to protein instability. Adapted from Lloyd et al. 2009 and Williams et al. 2009

### 1.6.2. BRCT domains

BRCT repeats were first discovered in BRCA1 (breast-cancer associated protein) (Bork et al., 1997) and since then have been shown to exist in a large number of proteins involved in the DDR (Bork et al, 1997). The BRCT domain encompasses 90-100 amino acids in length and can fold independently. The phosphopeptide binding activity of tandem BRCT domains was identified in BRCA1 and PTIP (PAX-interacting protein 1), protein in a genome-wide proteomics screen. This screen identified domains that specifically binded to a library of peptides centered on the motifs generated by ATM and/or ATR (Manke et al., 2003). Notable examples from DNA damage response that contain tandem BRCT repeats include BRCA1, MDC1, NBS1, 53BP1, PTIP, DNA ligase IV and TopBP1.

The phosphopeptide binding activity of BRCT domains is stronger for phosphoserine than for phosphothreonine. The phosphopeptide binding activity of BRCT domains is restricted to tandem BRCT repeats (Manke et al., 2003; Rodriguez et al., 2003). Furthermore, several BRCT domains were shown to strongly select for an aromatic residue in the pSer+3 position, e.g. BRCT domains of BRCA1 (Clapperton et al., 2004; Shiozaki et al., 2004) and MDC1 (Stucki et al., 2005). Interestingly, this +3 position is the site where many FHA domains exhibit strongest binding specificity, suggesting that other sequence elements like binding surfaces and protein conformations determine whether a given phosphorylation motif is recognized by another protein via FHA or (BRCT)2 domains (Reinhardt and Yaffe, 2013). Many structural studies revealed that the phosphopeptide-binding surface lies at the interface of the two BRCT domains, and each domain makes direct peptide contacts with the phosphopeptide ligand (Reinhardt and Yaffe, 2013). For example in the phosphopeptide from BACH1, the phosphoserine binds to a pocket in the N-terminal BRCT domain, whereas the Phe at the pSer+3 position binds to a deep groove at the interface between the two BRCT domains of BRCA1 (Clapperton et al., 2004). The γH2AX peptide also binds to a groove at the interface between the two BRCT domains of MDC1 (Stucki et al., 2005). Although the BRCT domains frequently appear as tandem pairs, which are separated by regions of different length; single BRCT domains are also found in DNA damage repair enzymes, such as REV1, XRCC1 and PARP1. MCPH1 carries a third BRCT domain at its N-terminus, PTIP has 6 BRCT domains and TopBP1 contains 8 BRCT domains (Reinhardt and Yaffe, 2013). However, not all BRCT domains act as phosphopeptide binding modules. Phospho-independent interactions of BRCT domains have been described between DNA ligase IV and as well as XRCC4, 53BP1 and p53 (Derbyshire et al., 2002; Sibanda et al., 2001)

The importance of BRCT domains in genomic integrity is highlighted by the cancer-associated mutations within the BRCT domains. For example, mutations in the BRCT domains of BRCA1 have been identified in familial breast and ovarian cancer syndrome and mutations within the BRCT domain of MCPH1 are associated with autosomal recessive primary microcephaly (MCPH) and cancer development (di Masi et al., 2011). Remarkably, the modular domains like FHA and BRCT domains, are

sometimes considered “promiscuous” (Basu et al., 2008; Mesquita et al., 2011). This is because FHA and BRCT domains can interact with more than just one phosphorylated protein. A typical example is the BRCT domain of BRCA1, which forms three different complexes with Abraxas, BACH1 and CtIP (Huen et al., 2010).

## 2. Aims

NBS1 is the least understood component of the MRN complex. All the studies that have been done on NBS1 are primarily based on NBS1 patient derived cell lines. Most of NBS patients harbor a *657del5* (frame shift mutation) that causes molecular scission of hNbs1- p95 into two halves. Structurally, 657del5 physically uncouples the FHA- and BRCT-mediated phosphoregulatory interactions of NBS1, from its MRE11 and ATM interaction regions. Such a genetic system is not well suited to study mutations, as complementing such a system essentially mean to study the effects of one mutation in the background of another mutation. Another model used to study NBS1 function was humanized mouse model, but this is again not a perfect system as it expresses a human protein in mouse cells. Although some studies have suggested the involvement of NBS1 in DNA end resection the precise function of NBS1 in this process in mammalian cells has not been investigated in detail. Furthermore, the functional relevance and contribution made by the FHA and BRCT domains of NBS1 in DNA end resection are not known. The aim of this PhD thesis was to rigorously test the role of NBS1 and its N-terminal FHA and BRCT domains in DNA end resection and G2M checkpoint activation and maintenance using a novel siRNA based complementation system. The specific aims thus were:

### 2.1. To generate and characterize a siRNA based complementation system for NBS1:

In an attempt to investigate the role of NBS1 in DNA end resection, we aimed to generate C-terminally myc-tagged, siRNA resistant constructs of wild type (WT) NBS1, R28A-NBS1 (FHA domain mutant), K160M-NBS1 (BRCT domain mutant), D.M.-NBS1 (FHA/BRCT domain mutant). The final goal was to generate a system, using siRNA resistant NBS1myc constructs and Flp-In T-REx system (Invitrogen) that allows controlled expression of the recombinant NBS1 and its FHA/BRCT mutants from an inducible promoter. We also intended to validate rigorously the NBS1 complementation system, as the rest of the studies designed for this PhD project were entirely dependent entirely on the robustness of this system.

**2.2. To investigate the role of the FHA/BRCT domains of NBS1 in DNA end resection:**

The MRN complex is essential for the sensing and signaling of DSBs, and in HR, by promoting the resection of DSBs in collaboration with CtIP. NBS1 was previously implicated in DSB repair by homologous recombination (HR) and DNA end resection. Thus, the second part of my thesis was focused on the analysis of human NBS1 and its FHA/BRCT mutants in DNA end resection. DNA resection is the first step in HR repair and it generates 3' ssDNA overhangs that are coated by RPA. The ssDNA overhangs can be visualized directly by neutral BrdU incorporation assay and indirectly by RPA and phospho RPA (S4/S8) foci formation. Using these markers as a read-out of resection, I tried to find out how NBS1 and its FHA and BRCT domains, contribute to I.R induced DNA resection.

**2.3. To study the role of NBS1 in DNA damage induced checkpoint maintenance:**

CtIP mediated DNA end resection has recently been implicated in G2M checkpoint maintenance. We reasoned that NBS1 should also be implicated in G2M checkpoint maintenance because it is essential for efficient DNA end resection as well. The aim of this study was to test this hypothesis by using NBS1 knockout cell line that was recently generated in our laboratory by the CRISPR/Cas9 technology.

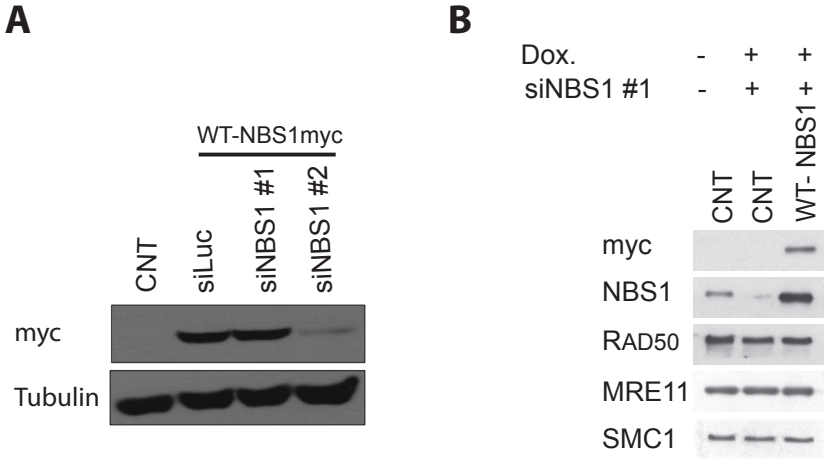
# 3. RESULTS

## 3.1 Rational for the development of a novel complementation system for human NBS1

The function of NBS1 in many aspects of the DDR has largely remained discordant. Conflicting findings have been reported regarding the role of NBS1 in ATM activation, cell cycle checkpoint regulation and DSB repair pathways. Elucidation of many important aspects of NBS1 has been obfuscated due to many reasons. The most problematic issue was the cell lines that have been used so far to study the cellular function of NBS1. These cell lines were derived from NBS patients. Approximately 90% of NBS patients are homozygous for the *657del5* allele, a mutation that causes molecular scission of the NBS1 protein. NBS cells express partially functional NBS1 in the form of N-terminal NBS1 fragment 26-kD (p26) and a C-terminal 70-kD NBS1 protein (p70) (Maser, 2001). To use these cells for the functional analysis of a specific mutation of NBS1 would essentially mean to study one mutation in the genetic background of another mutation of NBS1. A second issue is the embryonic lethality of null mutations of NBS1 in mammals (Kang et al., 2002) and inviability of mouse Nbs1-null mutants (Zhu et al., 2001). Finally, the phenotype of mouse models that have been used to study the role of NBS1 did not closely resemble the phenotype of NBS patients. A humanized mouse model that expresses hNbs1 657D5 in mNbs1$^{-/-}$ background has also been used to investigate the functional aspects of NBS1, which is again not a clean system, as NBS1 is the least conserved subunit of the MRN complex. Even though the functional domains are shared across the species, the sequences of mouse and human NBS1 are significantly different, so drawing direct parallels to make educated guesses about the role of functional domains has also remained a challenge. To understand the physiological role of NBS1 in the DDR and to assess the contribution of the FHA and BRCT domains of NBS1 for the overall role of NBS1 it was important to generate a 'clean' genetic system. This is the reason why we decided to develop a siRNA based complementation system for NBS1, which allowed us to perform a detailed structure/function analysis of the FHA and BRCT domains of NBS1 in the DDR.

### 3.1.1. Generation of the NBS1 complementation system

In order to study the physiological role of NBS1 in a clean genetic system, we developed a siRNA-based cellular complementation system, using the Flp-In T-REx system from Invitrogen in the human osteosarcoma cell line U2OS. The resistance against a siRNA targeting the NBS1 mRNA (siNBS1 #1), was introduced by making silent mutations in the NBS1 sequence, targeted by siNBS1 #1. The siRNA resistance of the WT-NBS1myc expression construct was tested both by DNA sequencing and western blotting. To test the siRNA resistance against siNBS1 #1, transient co-transfection of HEK-293T cells with an expression construct for wild type NBS1 (WT-NBS1myc expression plasmid in combination) with either with siNBS1 #1 or siNBS1 #2 was performed (see materials and methods). As expected WTNBS1-myc was expressed in the cells transfected with siNBS1 #1 but not in the cells with siNBS1 #2, suggesting that WT-NBS1myc was resistant to siNBS1 #1 (Figure 12A). Next, we asked if expression of WT-NBS1myc upon endogenous NBS1 depletion had any influence on the expression levels of the other components of the MRN complex, MRE11 and RAD50. Analyzing the expression levels of proteins by western blotting revealed that expression of WTNBS1-myc had no effect on the expression levels of the other subunits of the MRN complex (Figure 12B). Remarkably, it was also observed that the predominantly cytoplasmic localization of MRE11, which is a consequence of NBS1 depletion (Mirzoeva and Petrini, 2001), could be reversed upon expression of WTNBS1-myc, as revealed by immunofluorescence using antibodies against MRE11 (Figure 12C).



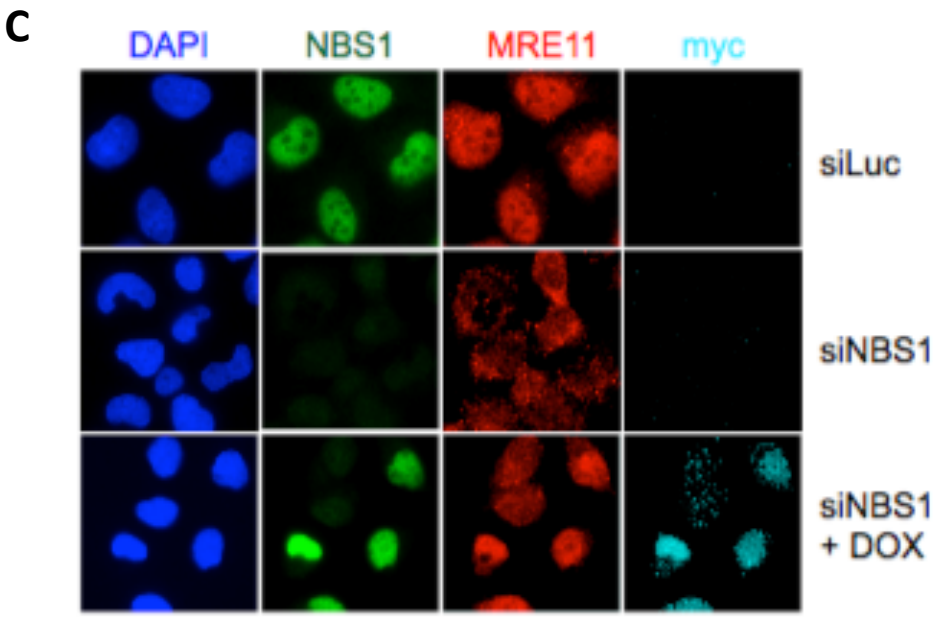



**Figure 12. siRNA-based complementation system for NBS1. (A)** HEK-293T cells were co-transfected with indicated siRNAs and WT-NBS1myc construct. 48 h after co-transfections, cells were lysed and whole cell extracts were analyzed by immunoblotting with the indicated antibodies. (**B**) Detection of expression levels of MRE11, RAD50 and NBS1 in WT-NBS1myc cell line 72 h after transfection with siNBS1. Expression of recombinant WT-NBS1myc was induced 60 h after siNBS1 transfection by the addition of 1 μM doxycycline (DOX) to the medium. **(C**) Immunofluorescence of the same cell line as in (A). Briefly, U2OS cell lines were transfected with the indicated siRNAs for 48 h before seeding cells on coverslips, followed by doxycycline treatment (1μg/ml) for 24 h. Cells were fixed and immunostained using anti-NBS1 antibodies (green) and anti-MRE11 antibodies (red). Nuclei were stained with DAPI (blue).

### 3.1.2. Validation of the NBS1 complementation system

Next, as a part of the characterization of the NBS1 complementation system, a colony formation assay was employed to determine if expression of WTNBS1-myc could rescue the increased IR sensitivity phenotype imposed by knockdown of endogenous NBS1. As expected, WT-NBS1myc rescued the radio sensitivity phenotype of NBS1 depleted cells, almost to the control levels (Figure 13).

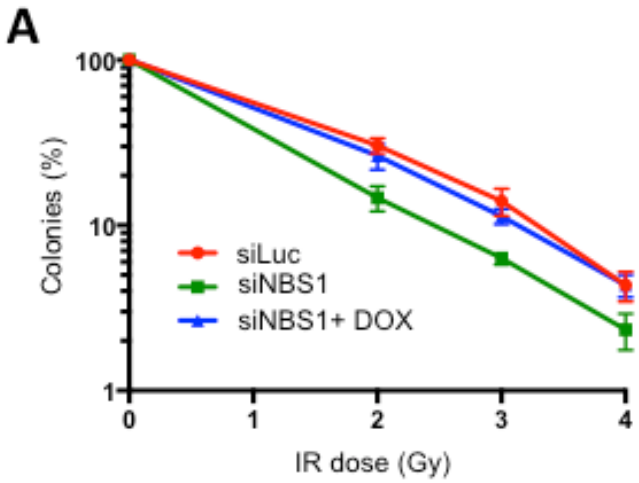



**Figure 13. Expression of wild type recombinant NBS1 rescues the radio-sensitivity phenotype of U2OS cells depleted from endogenous NBS1 by siRNA.** WT-NBS1myc cell line was transfected for 72 h with siNBS1 and expression of recombinant WT-NBS1myc was induced by the addition of DOX to the medium 60 h after siNBS1 transfection. Cell survival was determined after 12 days of irradiation with the indicated X-Ray doses, by colony-formation assay. Data are represented as mean ± SEM (n = 3 biological replicas)

Another important function of NBS1 is the recruitment of MRN complex to the sites of damage. Immunofluorescence analysis after laser micro-irradiation revealed that the recruitment of MRE11 to the DNA damage sites, which was abrogated on depletion of NBS1 from the cells was rescued upon expression of WT-NBS1myc (Figure 14).

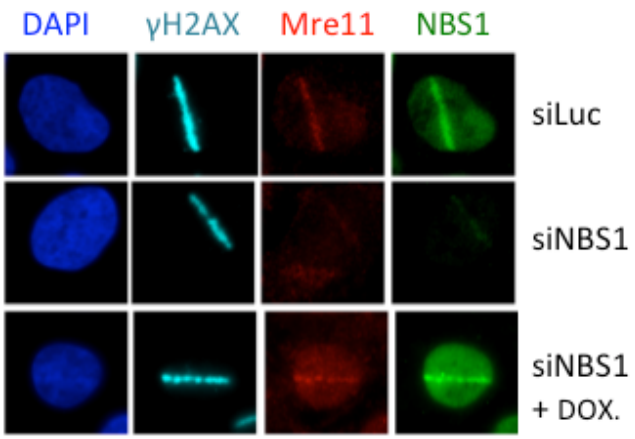


**Figure 14. Rescue of DNA damage induced recruitment of the MRN complex to sites of DSBs upon expression of WT-NBS1myc**. Control and WT-NBS1myc cell lines were transfected for 72 h with siNBS1 and expression of recombinant WT-NBS1myc was induced by the addition of DOX to the medium 60 h after siNBS1 transfection. Immunofluorescence of these cells after laser micro-irradiation revealed that WT-NBS1myc could rescue the recruitment of MRE11 to the sites of DNA damage.

NBS1 is phosphorylated at S343 by ATM in response to DSBs (Olson et al., 2007) and by ATR in response to replication stress (Yang et al., 2007). NBS1 phosphorylation at position S343 has been implicated in intra-S phase checkpoint activation (Lim et al., 2000). To ensure that WT-NBS1myc can take over all the functions of endogenous NBS1, it was also tested if the WTNBS1-myc is phosphorylated at S343. Western blot analysis of the protein lysates from cells that exclusively expressed WT-NBS1myc, using phospho-specific S343 NBS1 antibodies, revealed that WT-NBS1myc was indeed phosphorylated at S343 position (Figure 15). To test if the recombinant form of NBS1 forms an intact MRN complex with RAD50 and NBS1, co-immunoprecipitations (co-IPs) for the myc-tag of WT-NBS1myc were performed. WT-NBS1myc efficiently co-IPd with the other two components of MRN complex, MRE11 and RAD50 (Figure 16).

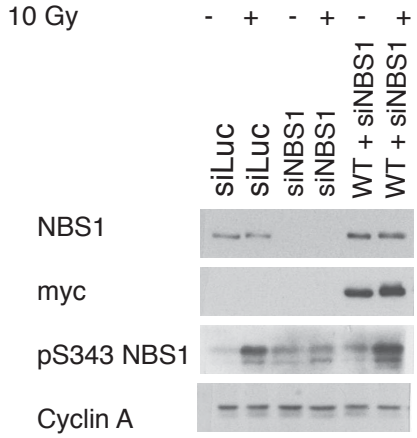



**Figure 15. WT-NBS1myc was phosphorylated at position S343.** Control and WT-NBS1myc cell lines were transfected for 72 h with siNBS1 and expression of recombinant WT-NBS1myc was induced by the addition of Doxycycline (DOX) to the medium 60 h after siNBS1 transfection. At this stage cells were irradiated with 10 Gy of IR and 1h later cells were harvested and lysed with SDS-lysis buffer for immunoblot analysis with the indicated antibodies.

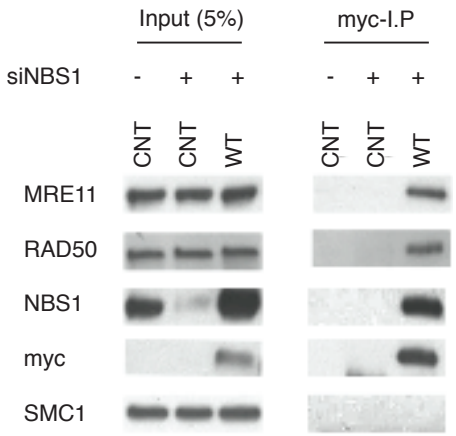



**Figure 16. Recombinant WT-NBS1myc is incorporated in the MRN complex.** NBS1-myc IPs in NBS1-myc U2OS Flp-In T-Rex cell lines. Briefly, control or WT-NBS1myc cells were treated with siNBS1 for 48 h and then induced with Doxycycline for 24 h. Cells were then lysed in NP-40 buffer and NBS1myc was immunoprecipitated from whole cell extracts using anti-myc antibody. The IPs were washed and analyzed by western blotting using the indicated antibodies.

When exposed to IR, mammalian cells arrest in G2 phase that occurs within an hour of exposure to ionizing radiation. This arrest is transient, with cells entering mitosis after a time that depends on the dose of IR. The G2M checkpoint prevents the cell with damaged DNA from entering mitosis. Activation of G2M checkpoint is characterized by a rapid reduction of the number of mitotic cells (Beamish et al., 1996; Xu et al., 2002) following treatment with IR. The activation of G2M checkpoint in cells is commonly measured by immunostaining with antibodies against phospho-Histone H3 (P-H3) that marks phosphorylation of histone H3 at serine 10, present in condensed chromosomes during mitosis. The percentage of cells in mitosis (termed the mitotic index) was measured by flow cytometry. It was observed that depletion of NBS1 partially alleviates the reduction of the mitotic index upon IR treatment, an indicator of impaired G2M checkpoint activation. However, expression of WTNBS1-myc could rescue the defect in activation of G2M checkpoint at low doses of irradiation (Figure 17). Based on all the above results, we concluded that most of the phenotypic consequences of lack of endogenous NBS1 are reverted by expression of WT-NBS1myc.

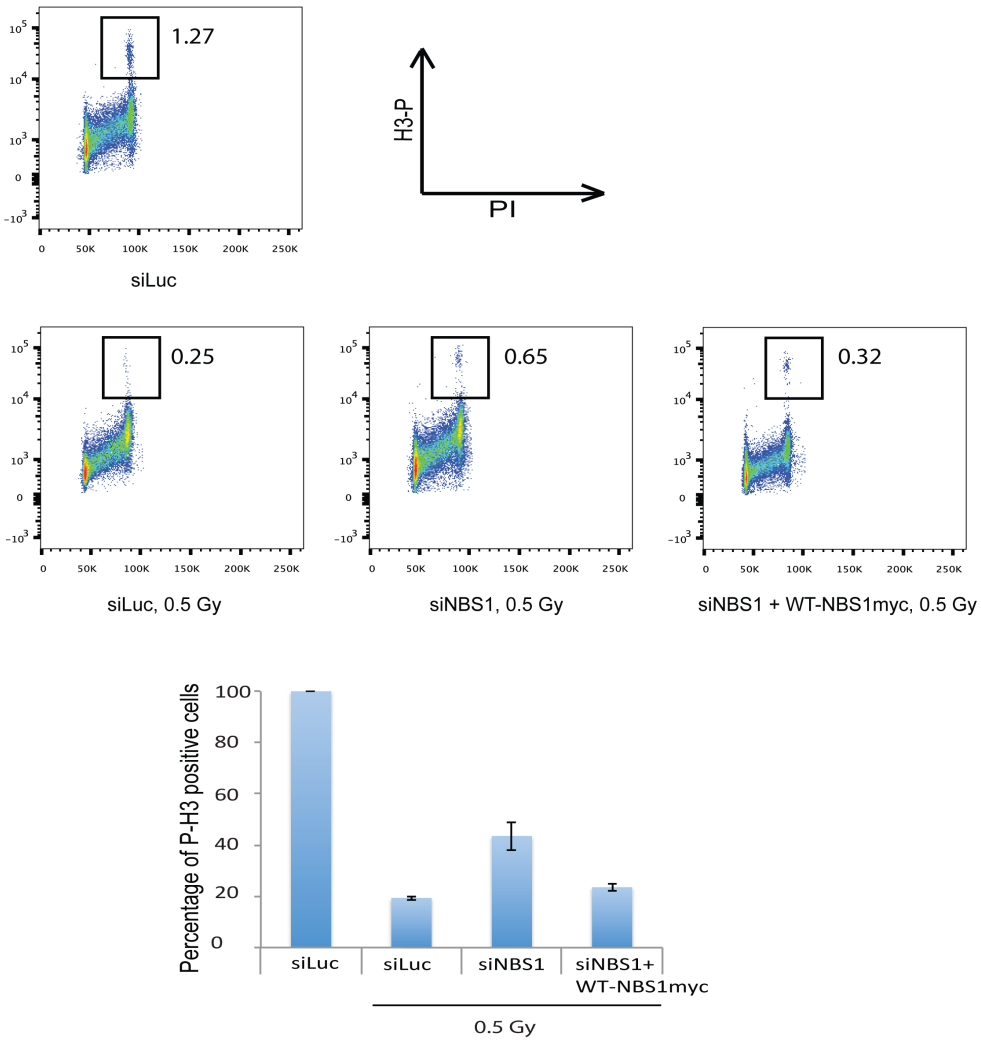



**Figure 17. Rescue of G2M checkpoint activation upon expression of WT-NBS1myc.** U2OS cells lines stably transfected with WT-NBS1myc, were treated with siNBS1 for 48 h and then induced with DOX for 24 h. Cells were harvested 1 h after 0.5 Gy of irradiation, fixed with 70% ethanol and stained with propidium iodide (PI) and an antibody against phosphorylated H3 (P-H3). The percentage of P-H3 positive cells was determined by flow cytometry. In this graph, three independent experiments are summarized. The bars represent the standard error of mean.

### 3.1.3. Generation and characterization of FHA and BRCT domain mutant cell-lines of NBS1

In order to perform structure/function analysis of the FHA and BRCT domains in the N-terminal region of NBS1, WTNBS1-myc construct was used as a base construct to introduce different site-directed mutations in order to substitute two amino acid residues that are conserved in the FHA and BRCT domains of NBS1. For disrupting the function of the FHA domain, Arg at position 28 was mutated to Ala (R28A) by site-directed mutagenesis. The x-ray crystal structure of the Rad53 FHA1 domain has revealed that this arginine is located in a peptide loop that binds phosphorylated Rad9 (Durocher et al., 2000; Durocher and Jackson, 2002). R28A mutation in the FHA domain of NBS1 is well characterized and has been used in many studies for the disruption of the phospho-binding function of the FHA domain (Cerosaletti and Concannon, 2003). To disrupt the BRCT domain of NBS1, Lys at position 160 was mutated to Met (K160M) by site directed mutagenesis. K160M mutation in the BRCT domain of NBS1 is also well characterized and it effects only the phospho-dependent interactions without impairing the solubility or structure of BRCT domain (Chapman and Jackson, 2008; Hari et al., 2010; Spycher et al., 2008).

Different FHA and BRCT domain mutant variants (Figure 18A) of NBS1myc were then used to generate inducible stable U2OS cell lines, using the same U2OS Flp-In T-REx system as described above. The expression of the doxycycline inducible NBS1myc forms: WT-NBS1myc, R28A-NBS1myc (FHA mutant), K160M-NBS1myc (BRCT mutant) and D.M.-NBS1myc (combined R28A/K160M double mutant), respectively was tested both by western blotting (Figure 18B) and by immunofluorescence (Figure 18C).

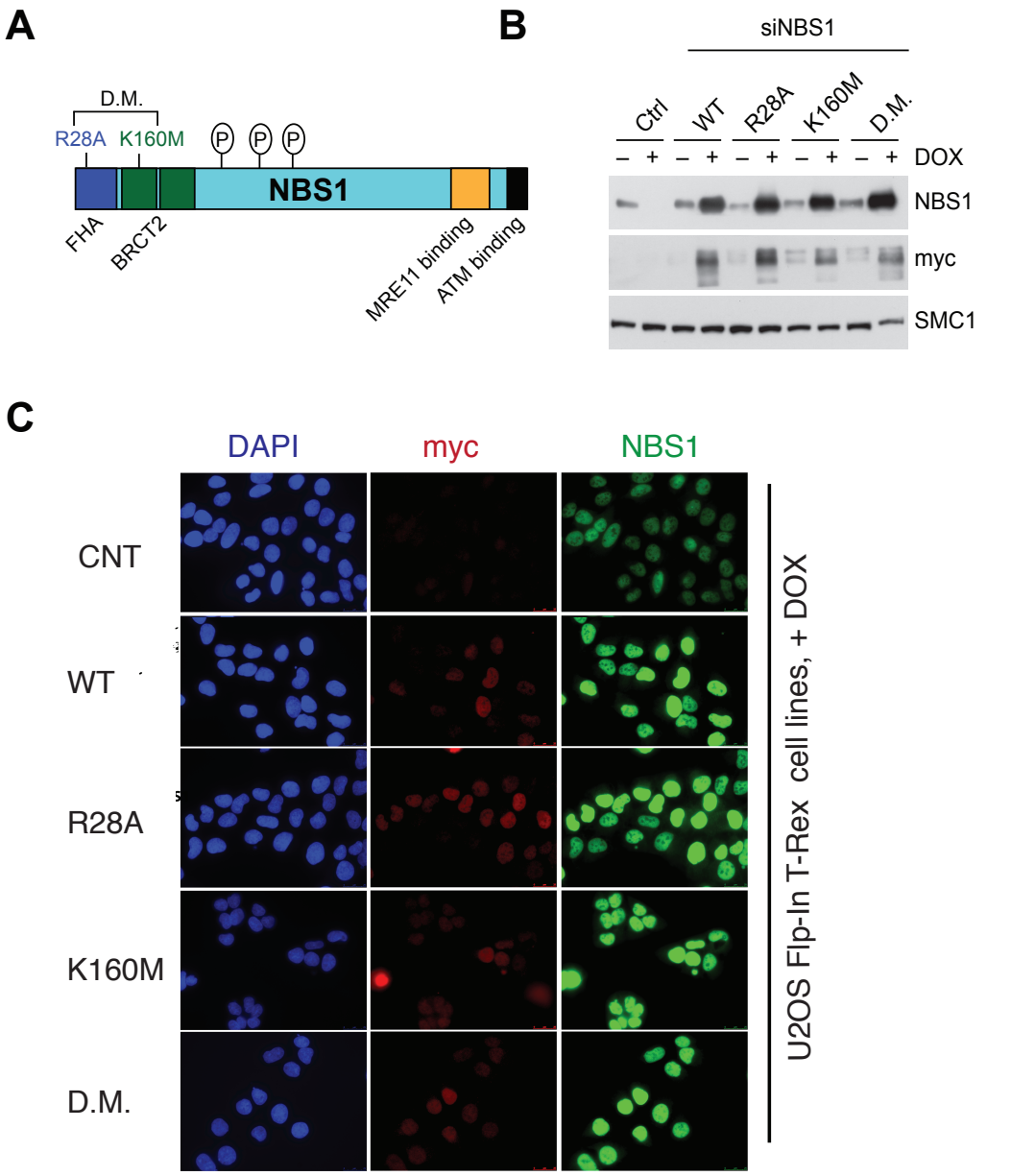



**Figure 18. Expression of FHA/BRCT domain mutants of NBS1. (A**) Schematic representation of the primary structure of NBS1. Location of mutations in the FHA domain and BRCT tandem domains are indicated. **(B)** Control or NBS1myc cells were treated with siNBS1 for 60 h and then induced with Doxycycline for 12 h. Cells were then lysed in SDS-lysis buffer. 50 µg of the protein extracts from the indicated samples were analyzed by western blotting using the indicated antibodies. Note that induction of expression of recombinant myc-tagged NBS1 by DOX leads to overexpression of NBS1. **(C)** U2OS cells lines were seeded cells on coverslips, followed by doxycycline treatment (1 µg/ml) for 24 h. Cells were fixed and immunostained using anti-NBS1 antibodies (green), anti-myc antibodies (red). Nuclei were stained with DAPI (blue). Induction of expression of recombinant myc-tagged NBS1, without knock-down of endogenous NBS1 by DOX leads to overexpression of NBS1.

Further the expression levels of the individual MRN subunits in the stable NBS1myc cell lines upon expression of R28A-NBS1myc, K160M-NBS1myc or D.M.-NBS1myc were tested. The levels of other subunits of MRN complex namely MRE11 and RAD50, essentially remained unaltered as measured by western blotting (Figure 19A). Next, it was tested if FHA and BRCT mutant forms of NBS1 form are incorporated into the MRN complex. To this end, co-IPs of the different FHA and BRCT mutants forms of NBS1 was performed in the stable cell lines using anti-myc beads and it was observed that R28A-NBS1myc, K160M-NBS1myc or D.M.-NBS1myc, efficiently interacted with MRE11 and Rad50 and thus are incorporated into the MRN complex (Figure 19B).

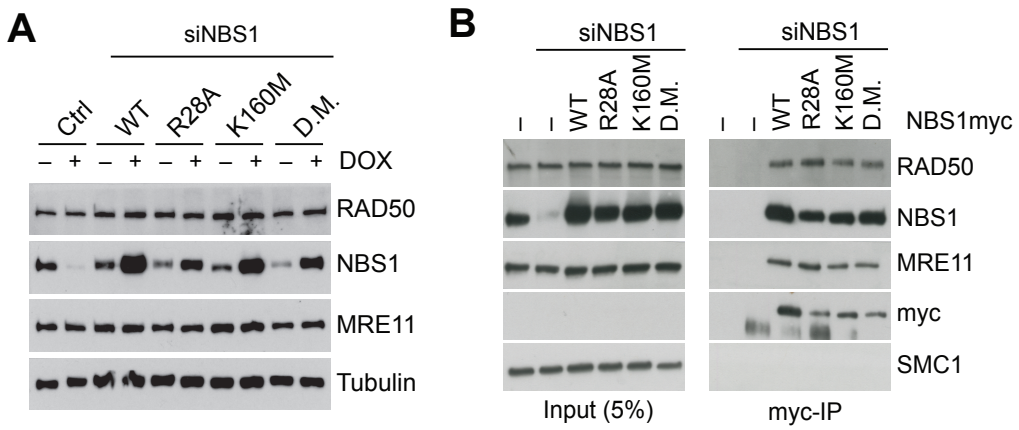



**Figure 19. (A) Expression of the recombinant NBS1 mutant proteins does not influence expression of RAD50 and MRE11.** NBS1-myc cell lines were treated with siNBS1 for 48 h and then induced with doxycycline for 24 h. Cells were lysed in SDS-lysis buffer and 50 µg of the protein from the indicated samples was analyzed by western blotting using the indicated antibodies. **(B) Recombinant myc-tagged NBS1 wild type and mutants exist in a complex with MRE11 and RAD50**. NBS1-myc IPs in U2OS: NBS1-myc cell lines. Cells were treated with siNBS1 for 48 h and then induced with doxycycline for 24 h. After harvesting, cells were lysed in NP-40 buffer and NBS1-myc was immunoprecipitated from whole cell extracts using anti-myc antibody beads. The immunoprecipitated proteins were washed and analyzed by western blotting using the indicated antibodies.

### 3.1.4. FHA and BRCT domain mutant forms of NBS1 are defective for phosphorylation at S343

As mentioned before, NBS1 phosphorylation at S343 in response to DSBs and replication stress is important for the function of NBS1 in DNA damage signaling and in intra-S phase checkpoint control (Buscemi et al., 2001). To test a potential role of FHA and BRCT domains on phosphorylation of NBS1 at S343, the FHA and BRCT mutant forms of NBS1-myc were expressed in the cell lines and endogenous NBS1 was down regulated at the same time. These cell lines were exposed to IR and western blot analysis with phospho-specific S343 NBS1 antibodies revealed that NBS1 S343 phosphorylation was partially impaired in FHA (R28A) and BRCT (K160M) mutants, while the FHA/BRCT (D.M.) mutant of NBS1 was completely defective in the phosphorylation of the S343 residue (Figure 20). This is in complete agreement with the previous studies that have implicated FHA and BRCT domains in phosphorylation of NBS1 on S343 (Zhao et al., 2002). Remarkably, we also observed that FHA and BRCT mutants were partially defective, while the D.M. was completely defective in the in the phosphorylation of S343 residue of NBS1.

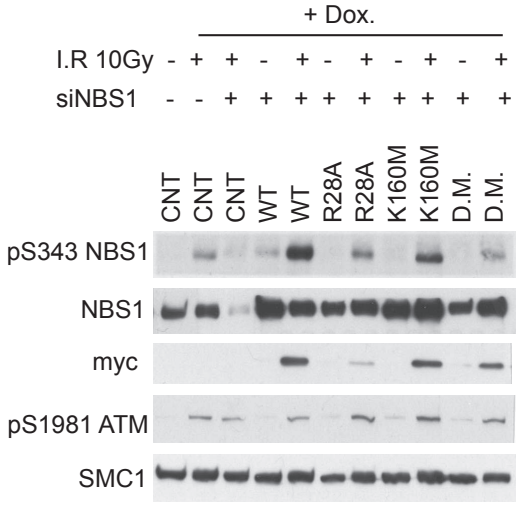



**Figure 20. Defective NSB1 S343 phosphorylation in FHA and BRCT mutants.** NBS1myc stable cell lines were transfected with siNBS1 for 60 h, after which they were induced with 1 μM DOX for 12 h. Cells were then exposed to 10 Gy of IR, recovered for 1 h and then lysed in SDS-Lysed buffer. 50 μg of the protein from the indicated samples was analyzed by western blotting using the indicated antibodies.

### 3.1.5. The NBS1 combined FHA/BRCT (D.M.) mutant is hypersensitive to IR

The very first studies that investigated the role of FHA and BRCT domains in cell survival reported that the BRCT domain of NBS1 and not the FHA domain of NB1 has a function in cell survival after IR (Zhao et al., 2002). To investigate the function of these domains of NBS1 in cell survival upon IR treatment in a clean genetic system, we performed clonogenic survival assays in our U2OS cell lines. By measuring colony formation 2 weeks after exposure of cells to a sub-lethal IR dose, a comparison of the different mutants in terms of survival after IR was performed. This analysis revealed that the individual FHA and BRCT domain mutants had little effect on cell survival after IR. However the combined FHA and BRCT mutant (D.M.-NBS1) expressing cells were as radiosensitive as the NBS1 depleted cells.

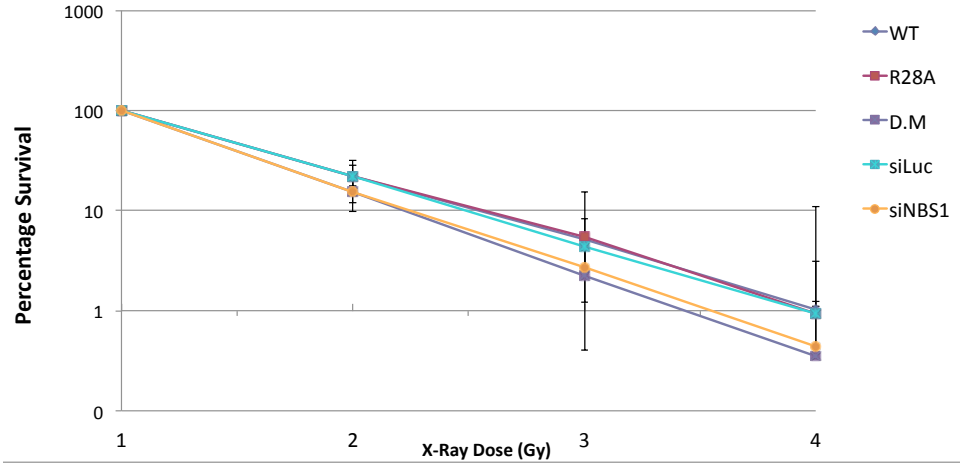



**Figure 21. Combined FHA/BRCT domain mutant is radiosensitive.** U2OS-NBS1myc cell lines were transfected for 72 h with siNBS1 and expression of recombinant WT-NBS1myc was induced by the addition of DOX 60 h after siNBS1 transfection. Cell survival was determined 2 days after irradiation with the IR doses, by the colony-formation assay. Plotted values represent average of 3 biological replicas.

## 3.2. Investigation of role of NBS1 in DNA end resection

The very first hints regarding the function of NBS1 in DNA end resection came from studies that implicated NBS1 in DNA repair by HR (Jazayeri et al., 2006; Tauchi et al., 2002). In fission yeast the FHA domain of Nbs1 interacts with Ctp1, via the motifs that are constitutively phosphorylated by CK2 (Lloyd et al., 2009; Williams et al., 2009). In mammalian cells, a recent study reported a similar mechanism, where NBS1 acts as a mediator of CtIP phosphorylation at T859 by ATM. However, contrary to previous reports this study suggested that FHA and BRCT domains of NBS1 interact with phosphorylated CDK sites in CtIP.

Recent work has also suggested that NBS1's role in DNA end resection is controlled by CDK activity. NBS1 S432 is phosphorylated by CDK in a cell cycle dependent manner and mutating this site in NBS1 disrupted the formation of IR-induced RPA foci and the generation of ssDNA at sites of DSBs (Falck et al., 2012). In contrast, another study also found that S432 in NBS1 is phosphorylated in a cell cycle dependent manner, but did not observe defective resection in the absence of NBS1 S432 phosphorylation (Wohlbold et al., 2012). The role played by NBS1 in DNA end resection is thus not clear yet and needs to be studied in a clean genetic system.

### 3.2.1. NBS1 plays an important role in DNA end resection

To investigate in detail the role of NBS1 in DNA end resection; U2OS, HEK-293T, MRC5 and HeLa cells were depleted of NBS1, by siNBS1 treatment and then exposed to irradiation. An hour after irradiation, cells were harvested and analyzed by Western blotting for signs of defective DNA end resection. In particular, phosphorylation of RPA at S4/S8 that is generally regarded as a surrogate marker for the generation of ssDNA at sites of DSBs (Sartori et al., 2007) was analyzed (Figure 22). Remarkably, knockdown of NBS1 by siRNA strongly compromised RPA2 phosphorylation in response to IR, and this was evident in all the cell lines that were tested. At the same time, cyclin A expression levels remained unchanged thus ruling out a cell cycle distribution effect of the RPA2 S4/S8 phosphorylation.

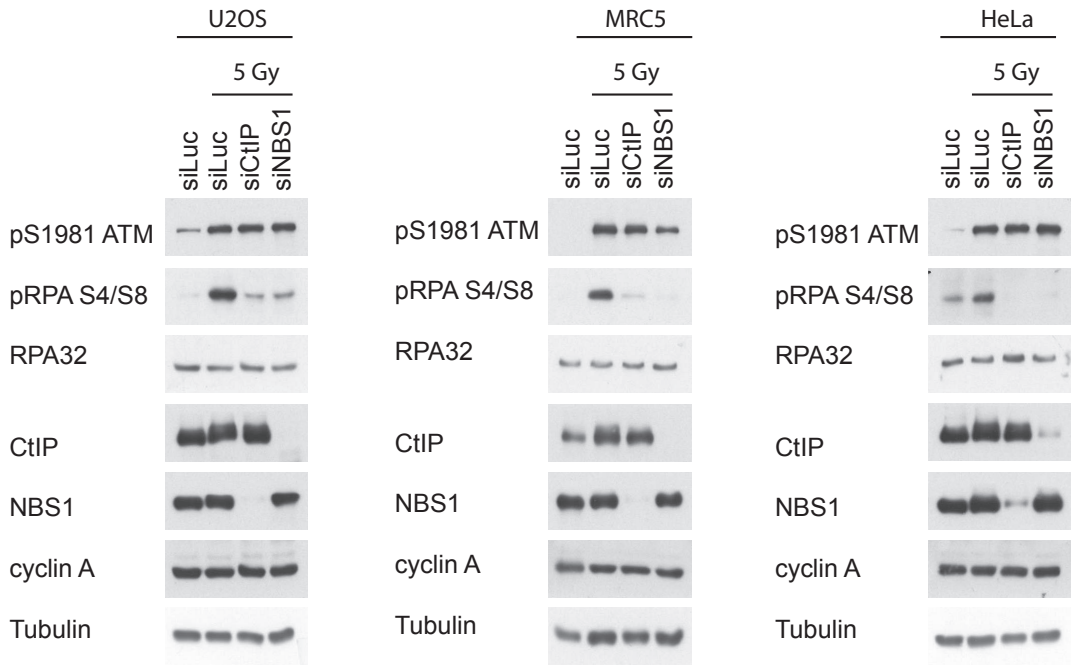



**Figure 22. Defective phosphorylation of RPA at S4/S8 upon NBS1 depletion.** U2OS, MRC5 and HeLa cells were transfected with indicated siRNAs and 72 h later, cells were treated with 5 Gy of IR, recovered for 1 h and then lysed in SDS-lysis buffer. 50 µg of the protein extracts of the indicated samples was then analyzed by western blotting using the indicated antibodies.

Next, DSB-induced RPA foci formation was investigated by immunofluorescence. For this, cells were grown on coverslips and irradiated with 5 Gy of IR. A mild pre-extraction protocol was applied to increase the signal/noise ratio. For quantification, random fields were recorded and the number of foci per cell was determined with the help of a self-written ImageJ macro (see Materials & Methods for detail). The distribution of number of RPA foci per cell was calculated and blotted with R Studio (ggplot2 library). As with CtIP depletion, NBS1 depletion strongly impaired DNA damage induced RPA foci formation (Figure 23). Cyclin A staining was used as a marker for S and G2 phase cells and it was observed that RPA foci were mostly formed in cells staining positive for cyclin A. Thus, in the quantitative image analysis (Figure 23B), only cyclin A positive cells were included.

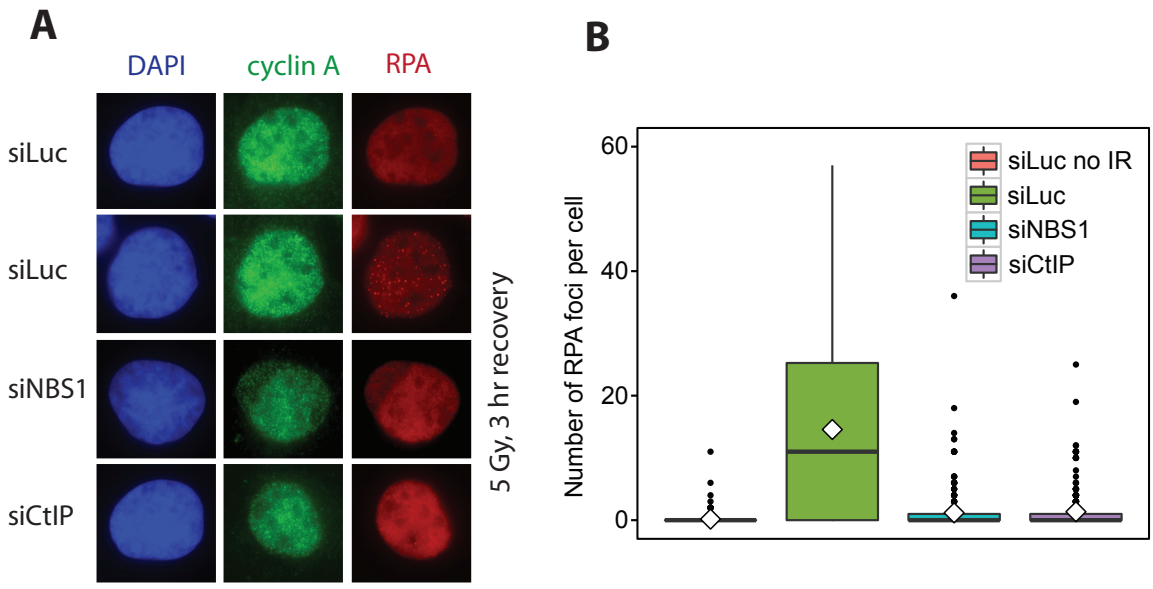



**Figure 23. Loss of RPA foci in NBS1 depleted cells. (A)** U2OS cells lines were transfected with the indicated siRNAs for 48 h and seeded on coverslips. 24 h later, cells were exposed to 5 Gy of IR, recovered for 3 h, fixed and immunostained using anti-RPA32 antibodies (red) and anti-cyclin A antibodies (green); nuclei were stained with DAPI (blue). **(B)** Quantification of RPA foci in NBS1-depleted cells. At least 100 cells per condition were analyzed by semi-automated image analysis (ImageJ macro). Only cyclin A positive cells were scored for RPA foci counting. Box plots represent the inter-quartile range (IQR) of the data (box), from the 25$^{th}$ percentile to the 75$^{th}$ percentile. The whiskers extend over 1.5 times the IQR in both directions. Black bar indicates the median and white diamonds indicates the mean of the distributions. Outliers are depicted by black dots (2 independent experiments, 1 representative experiment shown).

Next, RPA2 phosphorylation in response to IR was investigated by immunostaining of cells with anti-pRPA2 S4/S8 antibodies. As pointed out above, RPA2 S4/S8 phosphorylation serves as a marker for ssDNA generation at sites of DSBs. As observed with RPA foci, pRPA2 S4/S8 foci formation was also significantly reduced in NBS1 depleted cells (Figure 24A). Quantification of the pRPA pS4/S8 foci was done as described for RPA foci and the results revealed a very similar result: NBS1 depleted cells were almost as defective for RPA pS4/S8 foci formation as CtIP-depleted cells. RPA and pRPA foci formation are indirect markers for the generation of ssDNA at sites of IR-induced DSBs, as both signals indicate RPA binding to stretches of ssDNA and do not detect ssDNA *per se*. The ssDNA overhangs generated by resection can be visualized by incubating cells with the nucleotide analogue BrdU, which is incorporated into the DNA during normal DNA replication.

Immunofluorescence staining with a BrdU antibody under neutral conditions selectively recognizes BrdU incorporated in DNA only when the DNA is in the single-stranded form. Scoring ssDNA generation after IR treatment by a BrdU antibody again yielded very similar results as RPA and pRPA staining: namely defective ssDNA generation at sites of DSBs in NBS1 depleted cells very similar to CtIP depleted cells (Figure 24B and D). Taken together, by employing three different resection markers in quantitative immunofluorescence analysis it was revealed that NBS1 plays a very important role in DNA end resection and that its depletion by siRNA resulted in a resection phenotype that was almost as strong as the one caused by CtIP depletion.

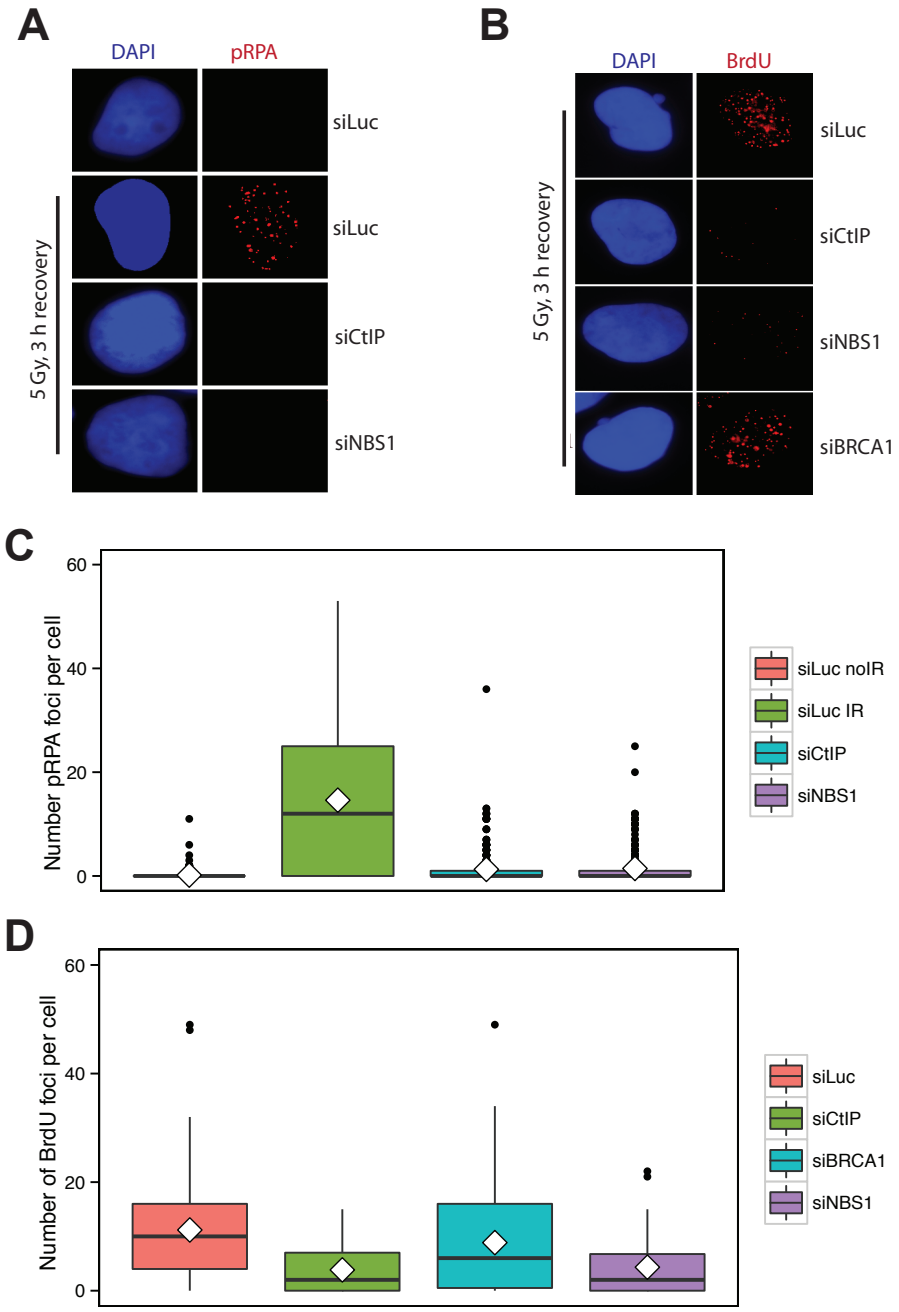



**Figure 24. (A)** Loss of pRPA foci in NBS1 depleted cells. U2OS cells lines were transfected with the indicated siRNAs for 48 h and seeded on coverslips. 24 h later, cells were exposed to 5 Gy of IR, recovered for 3 h, fixed and immunostained using anti-pRPA (S4/S8) antibodies (red); nuclei were stained with DAPI (blue). **(B)** Loss of BrdU foci in NBS1 depleted cells. U2OS cells lines were transfected with the indicated siRNAs for 48 h and seeded on coverslips, followed by addition of BrdU (10 μg/ml). 24 h later, cells were exposed to 5 Gy of IR, recovered for 3 h, fixed and immunostained with anti-BrdU antibodies (red); nuclei were stained with DAPI (blue). **(C)** Quantification of the number of pRPA foci per cell in NBS1-depleted cells. At least 100 cells per condition were scored by semi-automated image analysis (ImageJ macro). Box plots represent the inter-quartile range (IQR) of the data (box), from the 25$^{th}$ percentile to the 75$^{th}$ percentile. The whiskers extend over 1.5 times the IQR in both directions. Black bars indicate medians and white diamonds means of the distributions. Outliers are depicted by black dots. **(D)** Quantification of BrdU foci in NBS1-depleted cells as in B (2 independent experiments, 1 representative experiment shown).

### 3.2.2. Role of FHA and BRCT domains of NBS1 in DSB resection

Based on the observation that NBS1 depletion leads to a strong resection defect (see above), we next asked if the FHA and BRCT domains of NBS1 would contribute to NBS1's role in DNA end resection. These two domains control localization of NBS1 in the cell and connect it to other proteins such as MDC1 and TCOF1/Treacle (Hari et al., 2010; Larsen et al., 2014). Moreover, it was recently suggested that mutations in the FHA/BRCT region of NBS1 lead to a strong HR defect (Wang et al. 2013). In a first step it was important to know if expression of recombinant wild type NBS1 can rescue the defect in DNA end resection observed upon depletion of endogenous NBS1 by RNAi. Since a functioning complementation system for NBS1 had already been generated (see Section 1), RPA, pRPA and BrdU foci formation was tested by immunofluorescence analysis after IR treatment in NBS1-depleted cells in which the expression of recombinant WT-NBS1myc was induced by doxycyclin. WT-NBS1myc could rescue the ssDNA generation defect imposed by depletion of NBS1, thus ruling out the possibility that the defect in resection observed in NBS1 depleted cells is an artifact resulting from off-target effects of the siRNA used. Moreover, this observation also supported our notion that NBS1 has a very specific role in DSB end resection.

Next, we sought to investigate the contribution of the FHA and BRCT domains of NBS1 in DNA end resection. For this, RPA foci and pRPA foci were assessed after irradiation, in NBS1-depleted stable cell lines that inducibly expressed WT-NBS1myc, R28A-NBS1myc (FHA mutant), K160M-NBS1myc (BRCT mutant) and D.M.-NBS1myc (combined R28A/K160M double mutant), respectively. In a similar manner, the contribution of the FHA and BRCT domains of NBS1 in DNA end resection was analyzed by scoring ssDNA generation directly via BrdU staining. Remarkably, all three different resection based markers - RPA, pRPA and BrdU revealed that both the FHA and BRCT domains of NBS1 contribute in an additive manner to DSB end resection (Figure 25, Figure 26, Figure 27). The single FHA and BRCT domain mutants R28A-NBS1myc and K160M-NBS1myc were partially defective in resection, while the combined FHA/BRCT domain mutant (D.M.-NBS1myc) exhibited a very strong, robust defect in the generation of ssDNA at sites of DSBs. In all the assays it also emerged

that the BRCT domain mutant (K160M) was more defective in resection than the FHA domain mutant (R28A). The combined FHA/BRCT domain mutant (D.M.-NBS1myc) did not rescue the resection phenotype at all, suggesting that the function of NBS1 in DNA end resection is dependent on its N-terminal phospho-interaction modules.

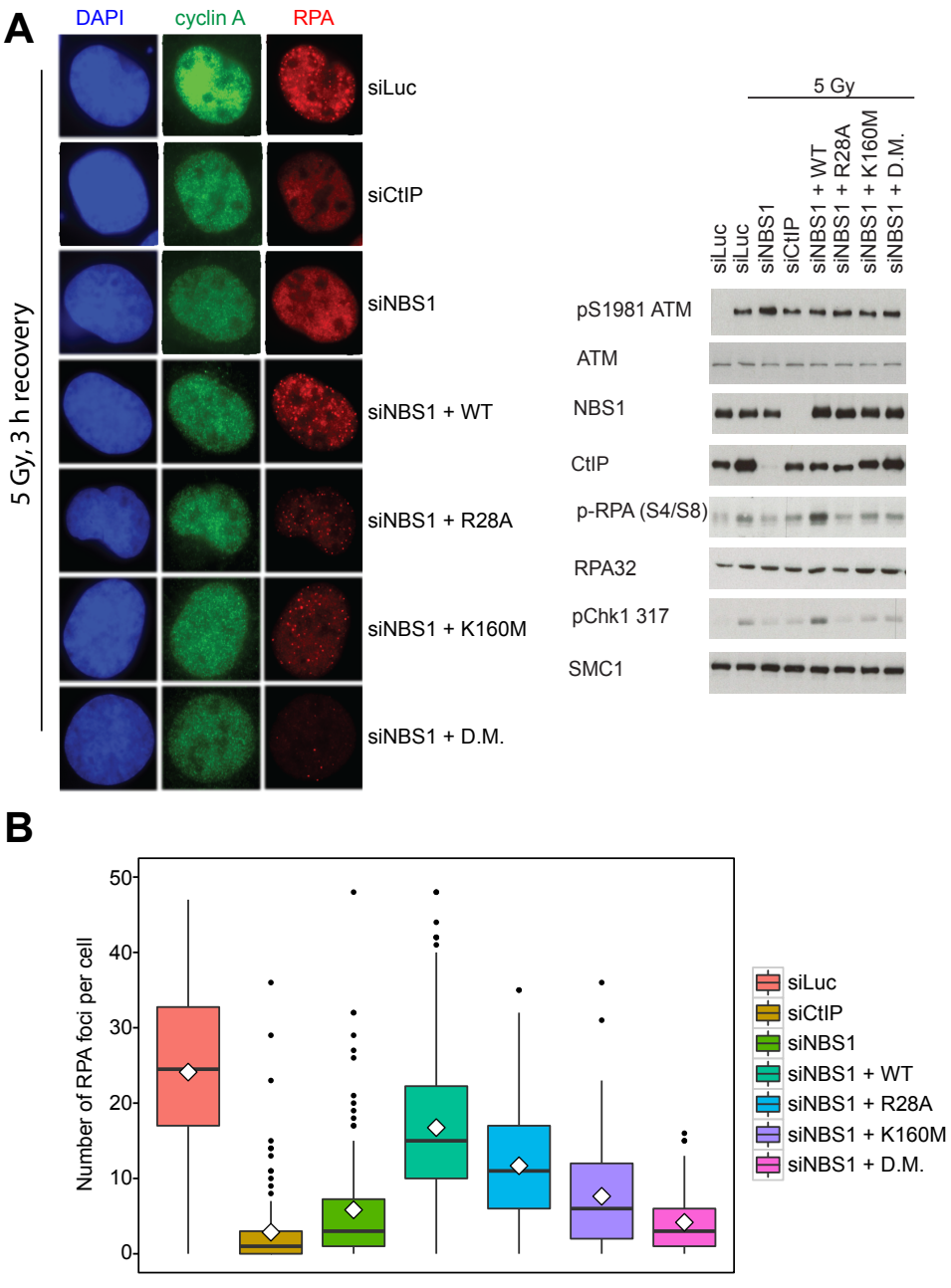



**Figure 25. Role of FHA and BRCT domains of NBS1 in RPA foci formation**. **(A)** U2OS Flp-In T-REx cell lines were transfected with the indicated siRNAs for 48 h before seeding cells on coverslips, followed by doxycycline treatment (1 μg/ml) for 24 h to induce FHA and BRCT mutant forms of NBS1. Cells were then exposed to 5 Gy of IR, fixed and immunostained for RPA (red) and cyclin A antibody (green); nuclei were stained with DAPI (blue). Same cells as in (A) were lysed in SDS-Lysis buffer and equal amounts of protein lysates from the indicated samples was then analyzed by western blotting

using indicated antibodies. **(B)** Quantification of RPA foci in U2OS Flp-In T-REx cells. At least 100 cells per condition were analyzed by semi-automated image analysis (ImageJ macro). Only cyclin A positive cells were considered for RPA foci counting. Box plots represent the inter-quartile range (IQR) of the data (box), from the 25$^{th}$ percentile to the 75$^{th}$ percentile. The whiskers extend over 1.5 times the IQR in both directions. Black bar indicates the median and white diamonds the mean of the data distributions. Outliers are indicated by black dots. (2 independent experiments, 1 representative experiment shown).

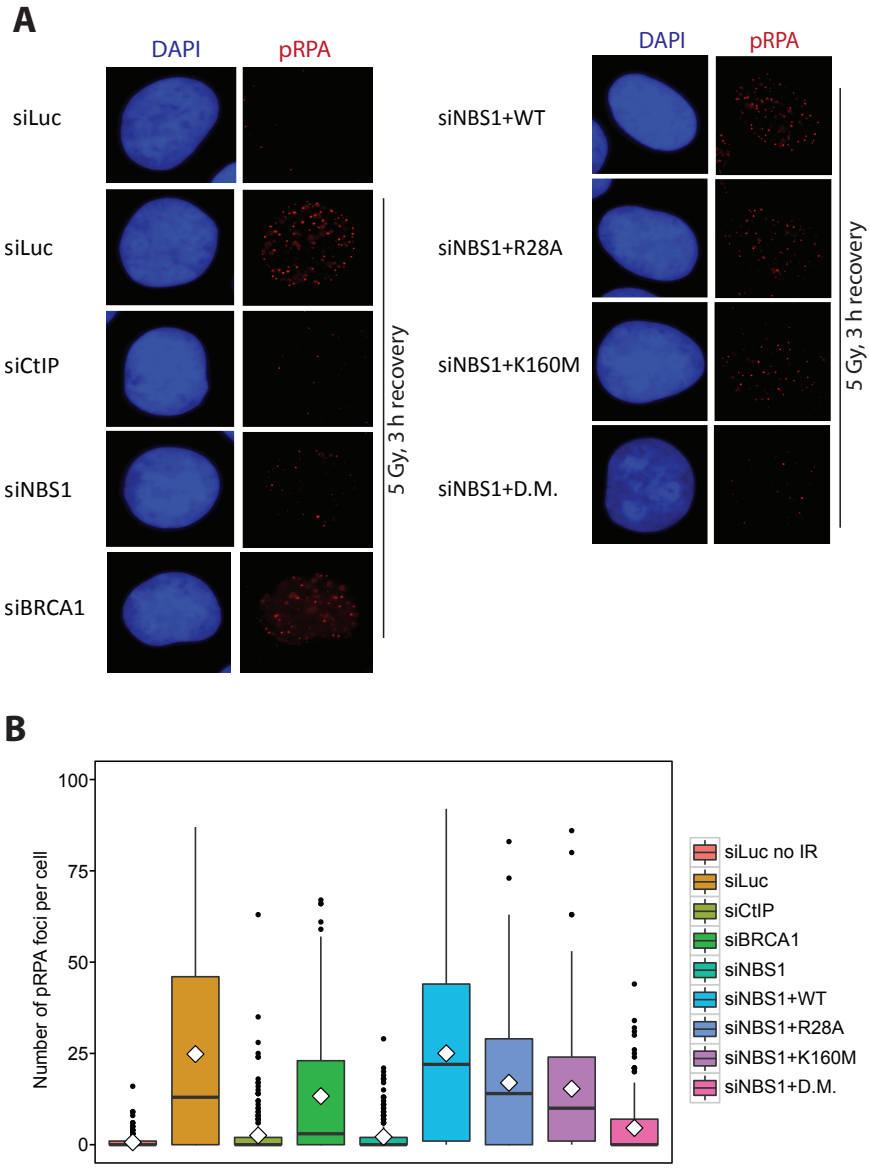



**Figure 26. Role of FHA and BRCT domains of NBS1 in pRPA foci formation**. **(A)** U2OS Flp-In T-REx cell lines were transfected with the indicated siRNAs for 48 h before seeding cells on coverslips, followed by doxycycline treatment (1 μg/ml) for 24 h to induce expression of FHA and BRCT mutant forms of NBS1. Cells were then exposed to 5 Gy of IR, fixed, pre-extracted and immunostained for pRPA S4/S8 (red); nuclei were stained with DAPI (blue). **(B)** Quantification of pRPA foci in U2OS Flp-In T-REx cells**.** CtIP depleted cells served as a positive control. At least 100 cells per condition were analyzed by semi-automated image analysis (ImageJ macro). Box plots represent the inter-quartile range (IQR) of the data (box), from the 25$^{th}$ percentile to the 75$^{th}$ percentile. The whiskers extend over 1.5 times the IQR

in both directions. Black bar indicates the median and white diamond the mean of the distribution. Outliers are indicated by black dots (2 independent experiments, 1 representative experiment shown).

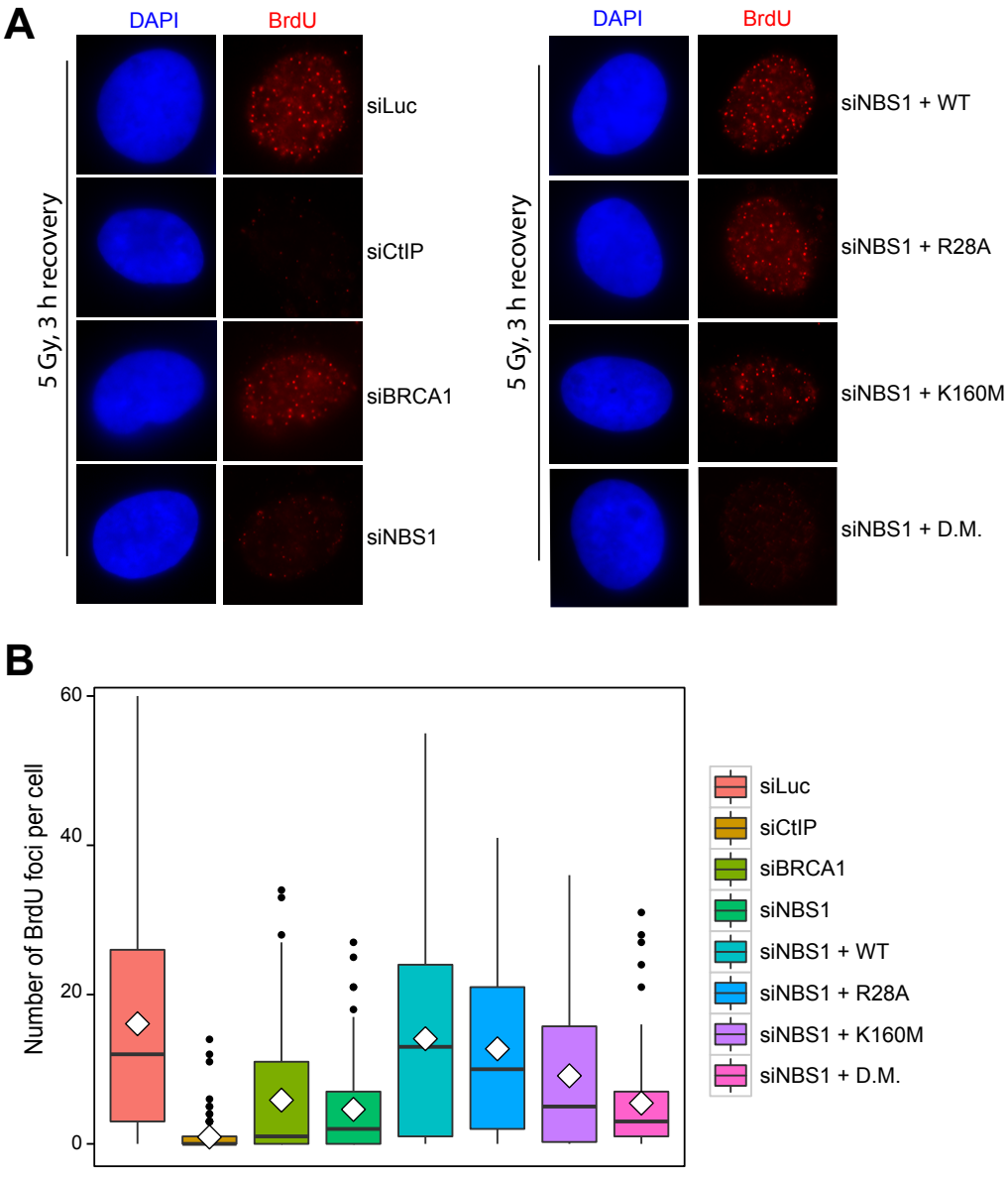



**Figure 27. Role of FHA and BRCT domains of NBS1 in BrdU foci formation**. **(A)** U2OS Flp-In T-REx cell lines were transfected with indicated siRNAs for 48 h before seeding cells on coverslips, followed by addition of BrdU (10 μg/ml) and doxycycline (1 μg/ml) for 24 h to label the DNA and induce the expression of FHA and BRCT mutant forms of NBS1. Cells were then exposed to 5 Gy of IR pre-extracted and immunostained for BrdU (red); nuclei were stained with DAPI (blue). **(B)** Quantification of BrdU foci in U2OS Flp-In T-REx cells. At least 100 cells per condition were analyzed by semi-automated image analysis (ImageJ macro). Box plots represent the inter-quartile range (IQR) of the data (box), from the 25$^{th}$ percentile to the 75$^{th}$ percentile. The whiskers extend over 1.5 times the IQR in both directions. Black bars indicate the median and white diamonds the mean of the distribution.

Outliers are indicated by black dots (2 independent experiments, 1 representative experiment shown).

As an alternative means to quantitatively assess the generation of ssDNA at sites of DSBs, a flow cytometry-based method analogous to the one described in (Forment et al., 2012) was adopted. The flow cytometry based quantification of ssDNA generation at sites of DNA damage worked efficiently when CPT was used as the DNA damage agent; however, this assay worked with a limited efficiency when IR was used as source of DNA damage (Figure 28).

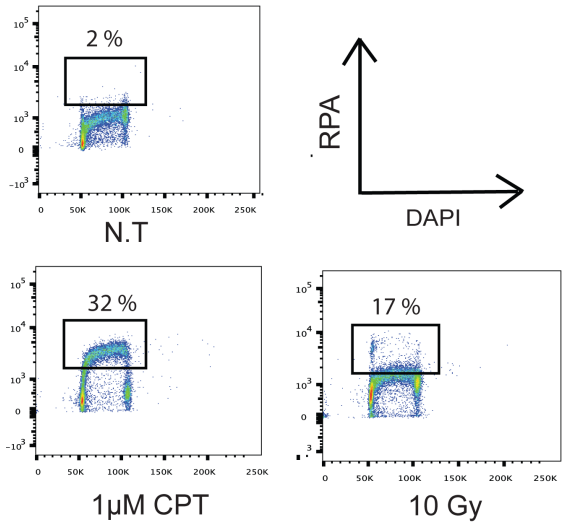



**Figure 28. Flow cytometry analyses of RPA levels in U2OS cells upon treatment with CPT and IR.** Briefly, U2OS cells were either treated with 1 μM CPT or with 10 Gy IR. After 2 h of exposure to DNA damage agents, cells were fixed, pre-extracted, permeabilized and then stained for RPA. It was observed that CPT treatment induced much higher RPA levels when compared to IR treatment (2 independent experiments, 1 representative experiment shown).

Despite this low efficiency we employed the cytometry-based assay to confirm our findings of the role of FHA and BRCT domains of NBS1 in DNA end resection by an alternative assay. EdU staining was used to gate for S phase cells and then RPA fluorescence quantification was done in the cells staining positive for EdU. Remarkably, as with immunofluorescence based foci formation assays, the single FHA (R28A) and BRCT (K160M) domain mutants of NBS1 were partially defective for DNA end resection. In addition, the double FHA and BRCT domain mutant (D.M.)

exhibited a very strong, robust RPA staining defect (Figure 29). In summary, the combined fluorescence microscopy and flow cytometry based analysis revealed that the FHA and BRCT domains of NBS1 are indispensible for DNA end resection and that they contribute in additive manner to the overall role of NBS1 in DNA end resection.

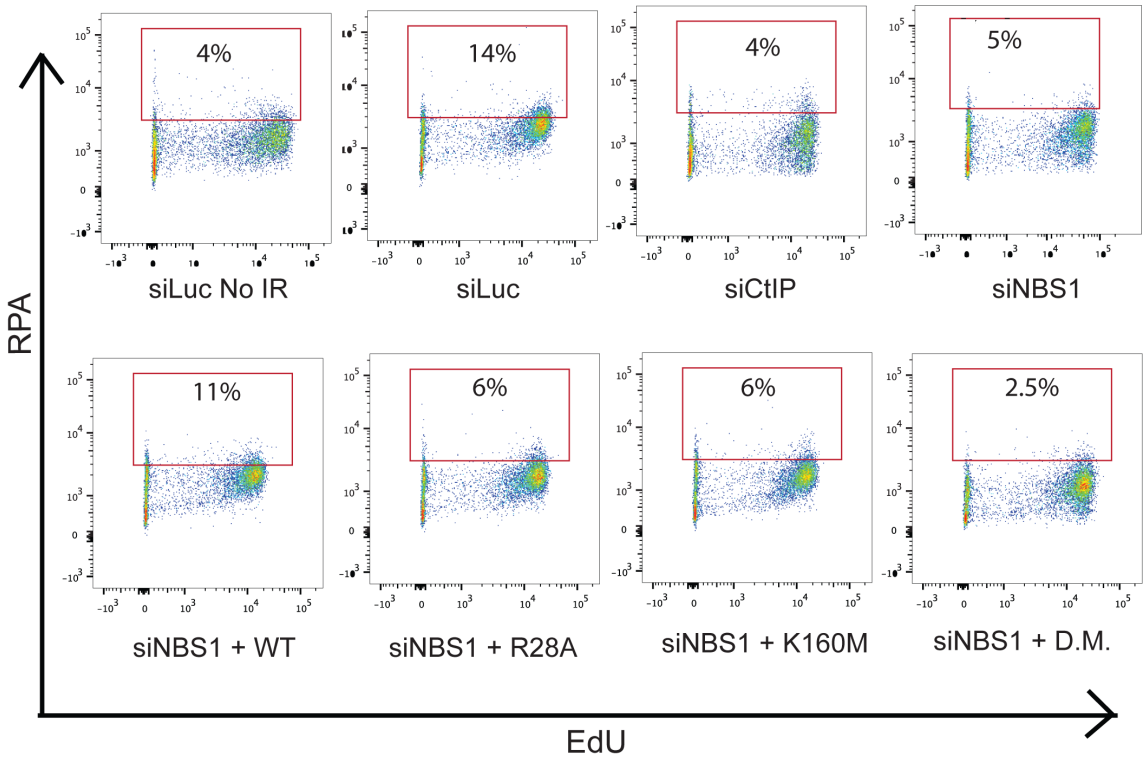



**Figure 29. Flow cytometry analyses of RPA and EdU florescence intensity in U2OS Flp-In T-REx cell lines.** Briefly, U2OS Flp-In T-REx cell lines were transfected with siRNA for 48 h before inducing them by addition of doxycycline (1 μg/ml) for expression of the indicated FHA and BRCT mutant forms of NBS1. Cells were then pulse-labeled with 10 μM EdU for 30 min before treating them with 10 Gy of IR. After 2 h cells were fixed, permeabilized and then stained with RPA antibodies. EdU detection reaction was carried out at this point, followed by DAPI staining. The red square marks the gate, counting the percentage of RPA positive cells. (2 independent experiments, 1 representative experiment shown).

### 3.2.3. Mechanistic explanation of defective DNA end resection phenotype in FHA and BRCT mutants of NBS1

To understand the mechanism of defective end resection in NBS1 mutants, we tested a couple of possibilities, which have been described in detail in the following sections.

#### 3.2.3.1 Normal nuclear localization of MRN complex in FHA and BRCT mutants

Since it was shown that NBS1 is crucial for both nuclear localization and formation of IR-induced foci (IRIF) of RAD50 and MRE11 (Zhao et al., 2002), we considered the possibility that the defect in resection observed upon expression of NBS1 mutants might be an indirect effect, caused by either cytoplasmic localization of MRE11 and RAD50 or by defective IRIF formation of MRE11 and RAD50 at sites of DSBs. To rule out the first possibility we examined the nuclear localization of MRE11 in the NBS1myc Flp-In T-REx cell lines. We observed that depletion of NBS1 by siRNA indeed resulted in predominantly cytoplasmic localization of MRE11 (Figure 30A). However, expression of a combined FHA/BRCT domain mutant form of NBS1 (D.M.) fully restored the localization of MRE11 to the nucleus. These results thus demonstrate that the resection defects of FHA and BRCT domain mutants of NBS1 are not caused by an inability to bring about MRE11 nuclear localization.

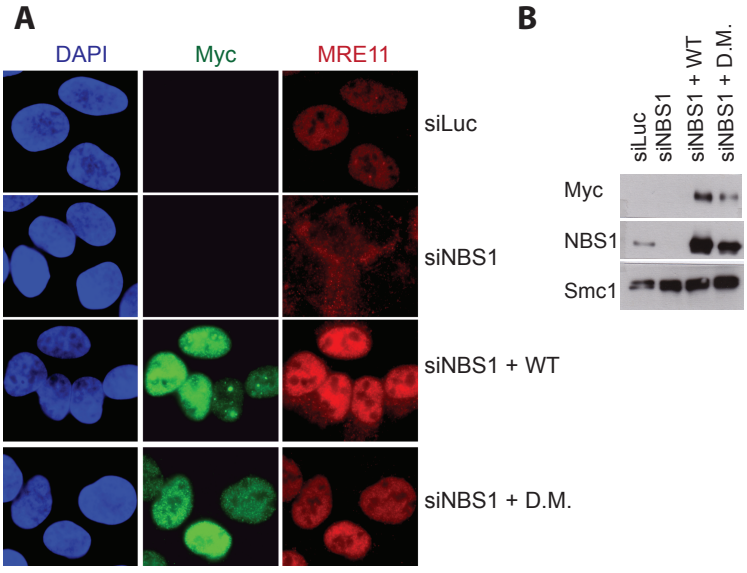


**Figure 30**. **Normal nuclear localization of MRE11 upon expression of D.M.-NBS1myc**. **(A)** Control, WT-NBS1myc cell line and D.M.-NBS1myc cell lines were transfected with siNBS1 for 60 h. and then the expression of indicated recombinant NBS1myc was induced by the addition of doxycycline (1 μg/ml) to the medium for 12 h. Immunofluorescence of these cells revealed that cytosolic localization of MRE11 in the absence of NBS1, could be rescued even on expression of recombinant D.M. NBS1myc. **(B)** Same cells as in (A) were lysed in SDS-Lysis buffer and equal amounts of protein lysates from the indicated samples was then analyzed by western blotting using indicated antibodies (2 independent experiments, 1 representative experiment shown).

#### 3.2.3.2. NBS1 functions in DNA end resection independently of MDC1

As a second possibility we reckoned that the resection defect of FHA and BRCT domain mutants of NBS1 might be caused by the defect in MRN IRIF formation observed with these mutants (Zhao et al., 2002). The MRN complex is recruited to sites of DSBs in an MDC1-independent manner, but its sustained interaction with the DSB-flanking chromatin requires the interaction of FHA/BRCT domains of NBS1 with MDC1 (Chapman and Jackson, 2008; Lukas et al., 2004a; Melander et al., 2008; Spycher et al., 2008; 2008) Thus, we considered the possibility that may be the sustained interaction of NBS1 with damaged chromatin is required for DNA end resection. Should this be the case we would expect to see an equally strong resection defect in MDC1 depleted cells as we see in NBS1 and/or CtIP depleted cells. However, quantitative analysis of RPA and pRPA foci formation revealed that DNA end resection is normal in MDC1 depleted cells (Figure 31). This also suggests that the role of NBS1 in DNA end resection is actuated by a different mechanism when compared to accumulation in IRIF at sites of DSBs and although this function of NBS1 relies on the FHA and BRCT domains; it is not dependent on the presence of MDC1 and on the phenomenon of MRN IRIF formation.

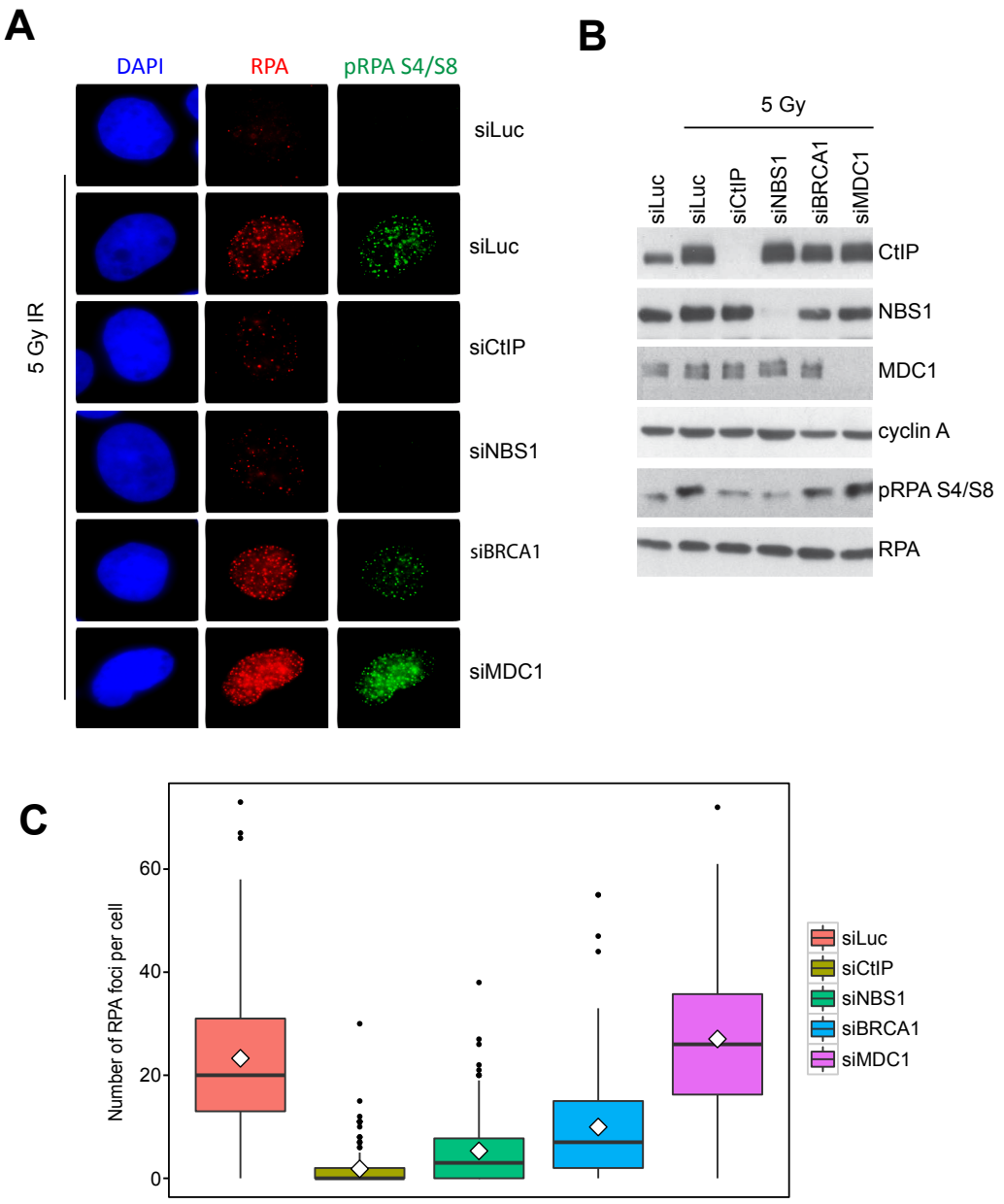



**Figure 31. NBS1's role in DNA end resection is independent of MDC1. (A)** No effect of MDC1 depletion on RPA and pRPA foci formation. U2OS cells were transfected with the indicated siRNAs for 48 h and then seeded on coverslips. 24 h later, cells were exposed to 5 Gy of IR, recovered for 3 h, fixed and stained with anti-RPA (red) and anti-pRPA S4/S8 antibody (green); nuclei were stained with DAPI (blue). **(B)** Normal phosphorylation of RPA at S4/S8 upon MDC1 depletion. U2OS cells were transfected with indicated siRNAs and 72 h later, cells were treated with 5 Gy of IR, recovered for 1hr and then lysed in SDS-Lysed buffer. 50 µg of the protein from the indicated samples was analyzed by western blotting using the indicated antibodies. **(C)** Quantification of RPA foci in U2OS cells. At least 100 control siRNA or siMDC1 treated cells were analyzed by semi-automated image analysis (ImageJ macro). Box plots represent the inter-quartile range (IQR) of the data (box), from the 25$^{th}$ percentile to the 75$^{th}$ percentile. The whiskers extend over 1.5 times the IQR in both directions. Black bar indicates the median and white diamonds indicate the mean of the distributions. Outliers are indicated by black dots (2 independent experiments, 1 representative experiment shown).

### 3.2.3.3. Rad17 is not involved in DNA end resection

A recent study suggested that the checkpoint factor Rad17 may facilitate MRN recruitment via the FHA/BRCT domains of NBS1 in the proximity of DSBs at an early time points after DNA damage infliction, which would impact DNA end resection and HR (Wang et al., 2014b). If that was indeed the case we would expect to see a resection defect in Rad17 depleted cells similar to the one observed upon expression of FHA and BRCT mutant forms of NBS1. To test the effect of Rad17 depletion on DNA end resection, we first analyzed RPA2 phosphorylation at S4/S8 phosphorylation in a western blot analysis, which revealed that there was no effect of Rad17 depletion on RPA2 phosphorylation (Figure 32). A quantitative analysis of BrdU and RPA foci upon IR treatment was also performed in cells depleted of Rad17 and it revealed no DNA end resection defect (Figure 33).

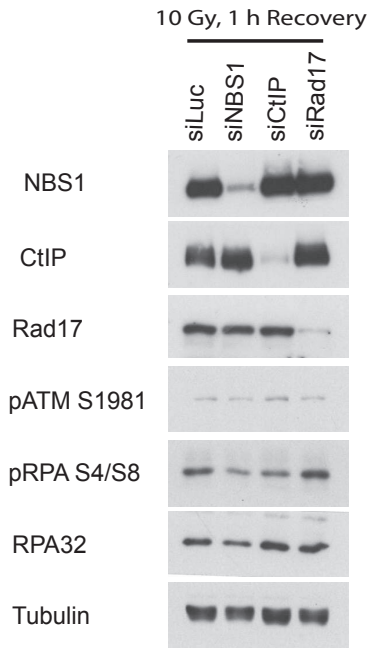



**Figure 32. Normal phosphorylation of RPA at S4/S8 upon Rad17 depletion.** U2OS, MRC5 or HeLa cells were transfected with indicated siRNAs and 72 h later, cells were treated with 5 Gy of IR, recovered for 1hr and then lysed in SDS-Lysis buffer. 50 µg of the protein from the indicated samples was then analyzed by western blotting using the indicated antibodies.

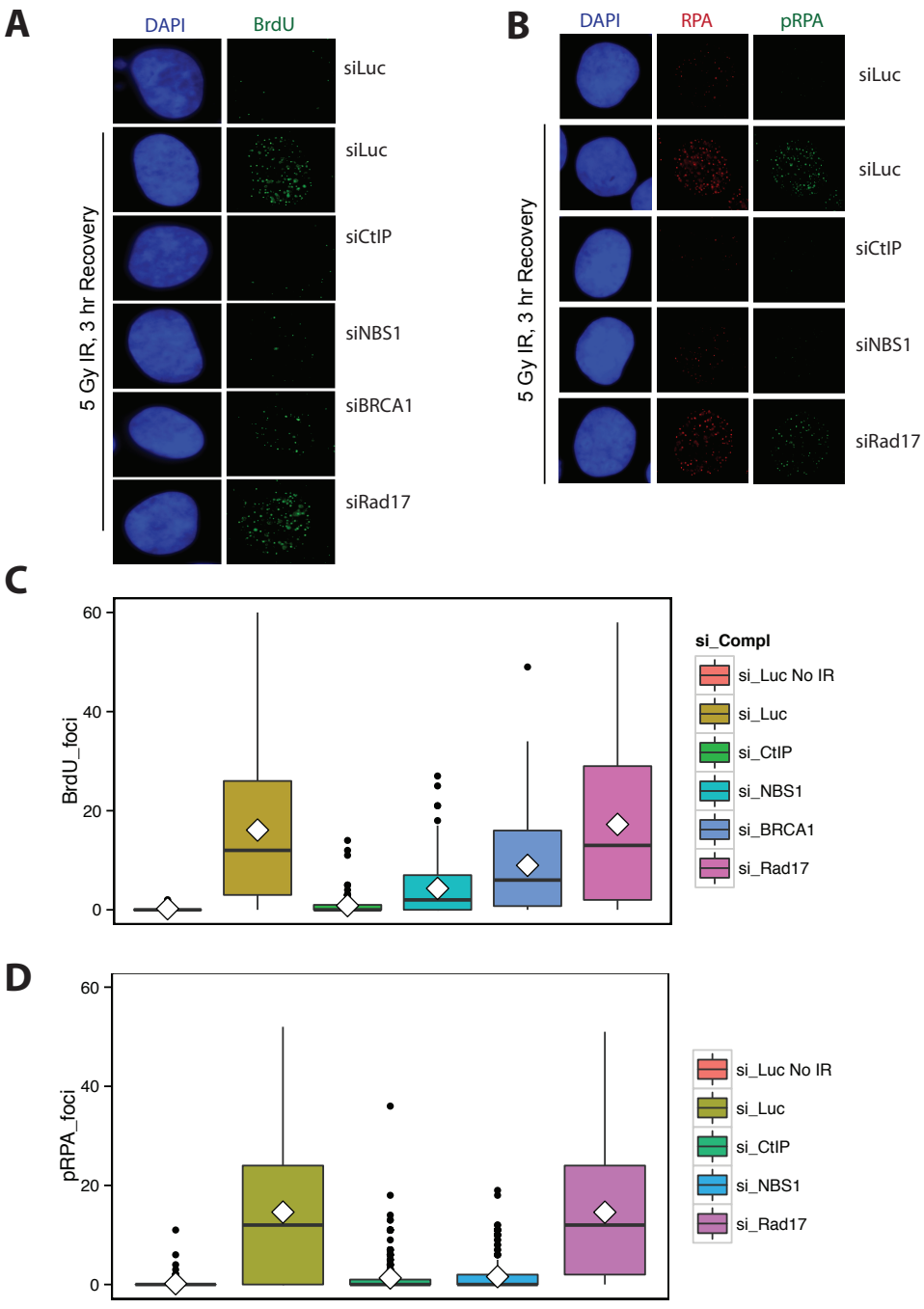



**Figure 33. Rad17 is not involved in DNA end resection. (A)** No effect of Rad17 depletion on BrdU foci formation. In short, U2OS cells were transfected with the indicated siRNAs for 48 h and then seeded on coverslips. 24 h later, cells were exposed to 5Gy of IR, recovered for 3 h, fixed and immunostained using anti-BrdU antibodies (green); nuclei were stained with DAPI (blue). **(B)** No effect of Rad17 depletion on RPA and pRPA foci formation. U2OS cells were transfected with the indicated siRNAs for 48 h and seeded on coverslips. 24 h later, cells were exposed to 5 Gy of IR, recovered for 3 h, fixed and immunostained using anti-RPA (red) anti-pRPA (S4/S8) antibodies (green); nuclei were stained with DAPI (blue). **(C)** Quantification of BrdU foci in Rad17 depleted U2OS cells. At least 100 control

siRNA or siRad17 treated cells were analyzed by semi-automated image analysis (ImageJ macro). Box plots represent the inter-quartile range (IQR) of the data (box), from the 25$^{th}$ percentile to the 75$^{th}$ percentile. The whiskers extend over 1.5 times the IQR in both directions. Black bars indicate the median and white diamonds the mean of the distributions. Outliers are indicated by black dots (2 independent experiments, 1 representative experiment shown). **(D)** Quantification of pRPA foci in Rad17 depleted U2OS cells as in C.

### 3.2.3.4. NBS1 phosphorylation at S432 is dispensable for DNA end resection

As mentioned before, two studies recently suggested a cell cycle regulated role of NBS1 in DNA end resection. In one of these studies it was shown that NBS1 is phosphorylated at S432 residue in a CDK dependent manner in the S and G2 phases of the cell cycle. Moreover, mutating this amino acid residue to Alanine disrupted the formation of IR induced RPA foci and the generation of ssDNA at sites of DSBs (Falck et al., 2012). In contrast, another study reported normal resection in the absence of NBS1-S432 phosphorylation (Wohlbold et al., 2012). The role of NBS1 phosphorylation at S432 in DNA end resection thus remains obscure. We speculated, if CDK dependent phosphorylation of NBS1 at S432 is implicated in DNA end resection then, FHA and BRCT domains of NBS1 might have a role in mediating CDK dependent phosphorylation of NBS1 at S432. Should this be the case, we would expect defective DNA end resection in cells that express a mutant form of NBS1 where Ser at position 432 has been changed to Ala (S432A). To test this possibility, we generated a cell line that stably expressed S342A NBS1myc in an inducible manner; using the U2OS Flp-In T-REx system in a similar manner as described before for FHA and BRCT mutants of NBS1. We then tested if exclusive expression of siNBS1 resistant S432A NBS1myc could rescue the resection defect observed in U2OS cells depleted of endogenous NBS1. BrdU, pRPA and RPA foci formation upon IR treatment in cells that express S3432A-NBS1myc was normal, thus revealing that phosphorylation of NBS1 at S432 is dispensable for DNA end resection in U2OS cells (Figure 34).

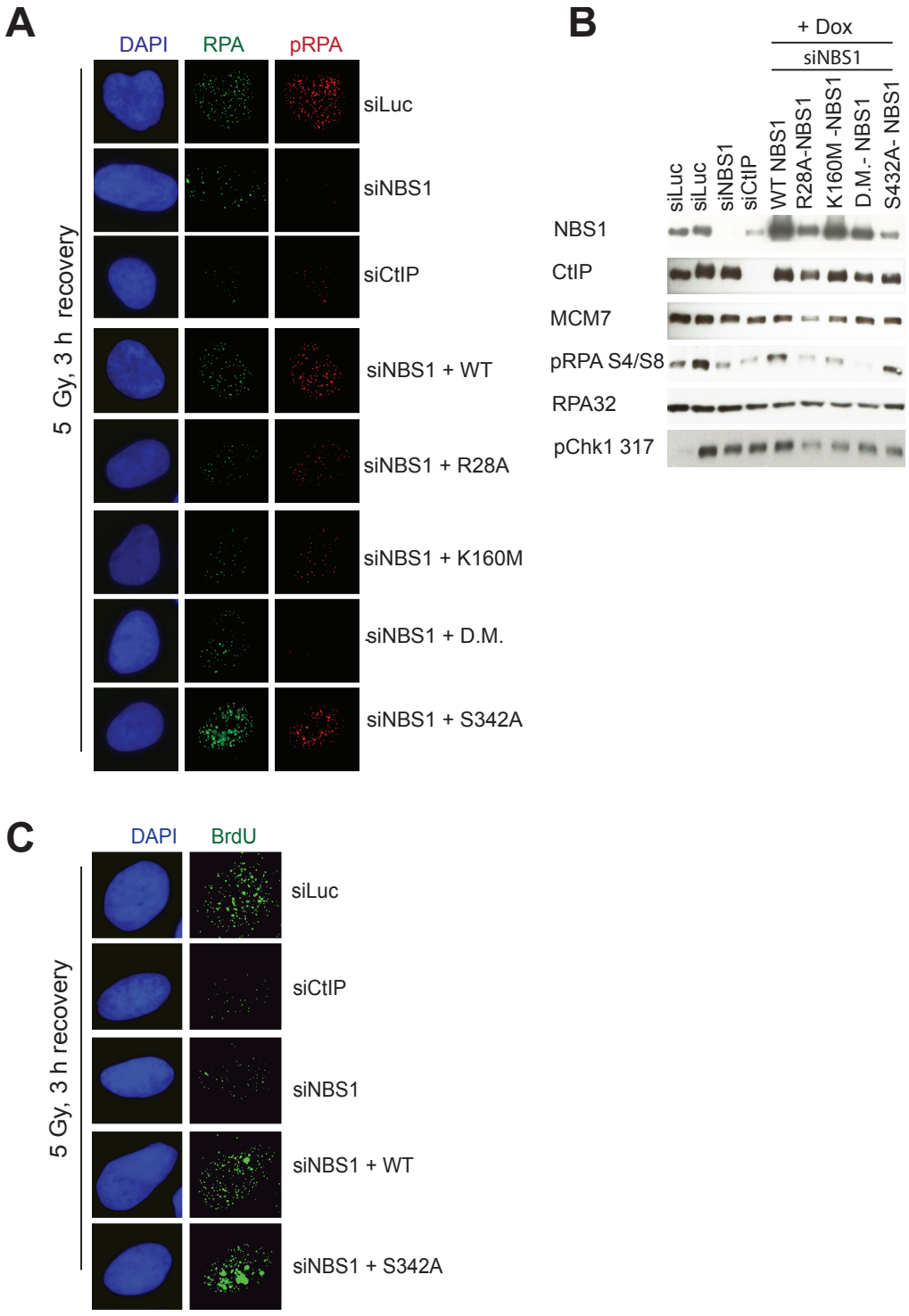



**Figure 34. NBS1 pS342 is dispensable for DNA end resection. (A)** Normal RPA and pRPA foci formation upon expression of S432A NBS1myc. U2OS Flp-In T-REx cells lines were transfected with the indicated siRNAs for 48 h and seeded on coverslips. 24 h later, cells were exposed to 5 Gy, recovered for 3 h, fixed and immunostained using anti-RPA (green) and anti-pRPA S4/S8 antibodies (red); nuclei were stained with DAPI (blue). **(B)** Normal RPA phosphorylation at S4/S8 upon expression of S432A NBS1. Briefly, U2OS Flp-In T-REx cell lines were transfected with siNBS1 for 48 h before inducing the expression of indicated mutant forms of NBS1 by addition of doxycyclin. Cells were then exposed to 5 Gy of IR, recovered for 1hr and lysed in SDS-lysis buffer. 50 µg of the protein from the indicated samples was analyzed by western blotting using the indicated antibodies (2 independent

experiments, 1 representative experiment shown) **(C)** Normal BrdU foci formation upon expression of S432A NBS1. U2OS Flp-In T-REx cells lines were transfected with the indicated siRNAs for 48 h and seeded on coverslips. 24 h later, cells were exposed to 5 Gy of IR, recovered for 3 h, fixed and immunostained with anti-BrdU antibodies (green); nuclei were stained with DAPI (blue); (2 independent experiments, 1 representative experiment shown).

### 3.2.3.5. NBS1 interacts with CtIP via FHA and BRCT domains

In a further quest to find a possible mechanistic explanation of the effect of FHA and BRCT domains of NBS1, we speculated that defective end resection in NBS1 mutants might be the outcome of loss of interaction with some key player of DNA end resection. Many studies have reported that CtIP interacts with MRN, although the mechanism of this interaction is not clear. However, a more direct role of NBS1 in DNA end resection was implied when it was shown that interaction between NBS1 and CtIP is implicated in DNA damage resistance (Lloyd et al., 2009; Williams et al., 2009). However, it remained unknown if a similar mechanism of interaction exists between CtIP and NBS1, exists in mammalian cells also and if this interaction has a role in DNA damage response. Another important question that also has not been addressed so far is whether the interaction between CtIP and NSB1 has a role in DNA end resection.

Although multiple point of interaction have been suggested between NBS1 and CtIP (Wang et al., 2013a; Yuan and Chen, 2009), we were particularly interested in dissecting the mode of interaction between NBS1 and CtIP; and to understand if this interaction is implicated in DNA end resection. To specifically investigate the direct interaction between NBS1 and CtIP and to separate this interaction from the interaction between RAD50 or MRE11 and CtIP we overexpressed different FHA and BRCT domain mutants of Flag-tagged N-terminal fragment (1-382 aa) of NBS1 that lacked any MRE11 interaction site in HEK 293T cell lines and performed Flag-tag pull downs. Analysis of pulled down fractions by western blotting revealed no traces of CtIP (Figure 35), even in WT-NBS1 (N-terminal) pull-down fraction. However, we observed that WT-NBS1 N-terminal fragment interacted in a DNA damage dependent manner with BRCA1 and this interaction was lost in the FHA and BRCT domain mutants of NBS1. Based on these results and the previous studies, which

implied that in vivo interaction of NBS1 with CtIP is strengthened when NBS1 forms a complex with MRE11 and Rad50 (Chen et al., 2008); we concluded that the interaction between N-terminal part of NBS1 with CtIP was not strong enough to be captured by immunoprecipitation.

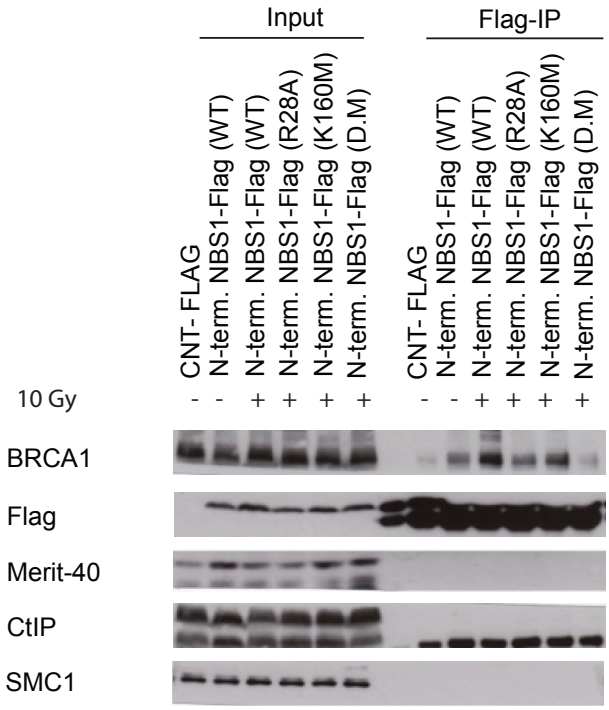



**Figure 35. An N-terminal fragment of NBS1 (1-382 aa) does not interact with CtIP.** 293-T cells transfected with Flag tagged N-terminal NBS1 fragments (wild type and R28A, K160M or R28A/K160M (D.M.) were lysed and Flag-NBS1 fragments were immunoprecipitated from whole cell extracts using anti-Flag antibody beads (M2). The immunoprecipitated proteins were then analyzed by western blotting using indicated antibodies.

Because no interaction between an N-terminal fragment of NBS1 (containing the FHA/BRCT region) and CtIP could be detected, we performed co-immunoprecipitation experiments with overexpressed full-length NBS-myc in the U2OS Flp-In T-REx cell lines. A very weak interaction between WT-NBS1myc and endogenous CtIP was observed (Figure 36). A very faint band in control myc IP was present, which implied that non-specific interactions between endogenous CtIP and the anti-myc beads take place. Based on these results, we concluded that the NBS1 and CtIP interaction may be too weak for detection with endogenous proteins and that the overexpression of both NBS1 and CtIP might be necessary to capture a specific interaction by immunoprecipitation.

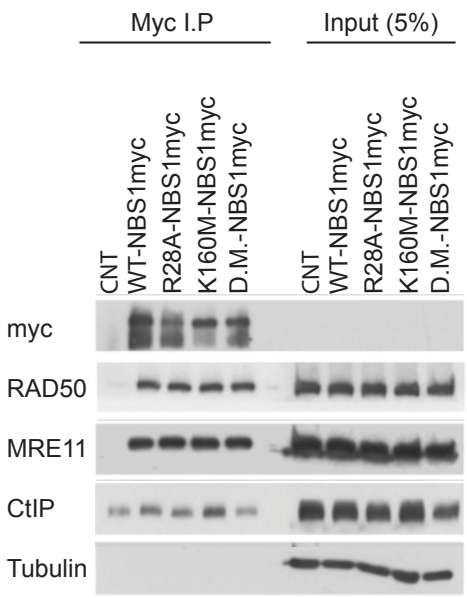



**Figure 36. WT-NBS1myc interacts with endogenous CtIP.** Briefly, U2OS Flp-In T-REx cell lines were transfected with siNBS1 for 48 h before inducing recombinant protein expression by the addition of doxycycline (1 μg/ml). Cells were lysed and NBS1-myc was immunoprecipitated from whole cell extracts using beads coated with anti-myc antibodies. The immunoprecipitated proteins were analyzed by western blotting using the indicated antibodies (2 independent experiments, 1 representative experiment shown).

In order to detect a robust interaction between NBS1 and CtIP we over-expressed full length Flag-CtIP and different myc-tagged full-length FHA and BRCT domain mutants of NBS1 in HEK-293T cell by co-transfection. Co-immunoprecipitaiton experiments revealed that WT-NBS1myc interacted with Flag-CtIP but single FHA and BRCT domain mutants namely R28A-NBS1myc and K160M-NBS1myc as well as D.M.-NBS1myc were completely defective for CtIP interaction (Figure 37). Taken together, these results suggest that CtIP and NBS1 interaction requires full-length NBS1 and this interaction is dependent on the FHA and BRCT domains of NBS1. Remarkably, we also noticed an enhanced CtIP interaction with MRE11 and RAD50 in cells transfected with WT-NBS1myc but not in cells transfected with FHA and BRCT domain mutants of NBS1. This again confirms that FHA and BRCT domains of NBS1 are important for strengthening the interaction between CtIP and MRN complex.

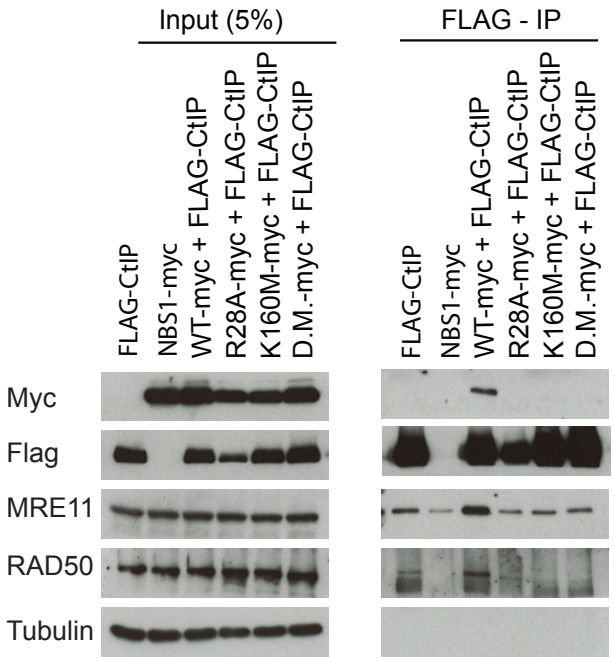
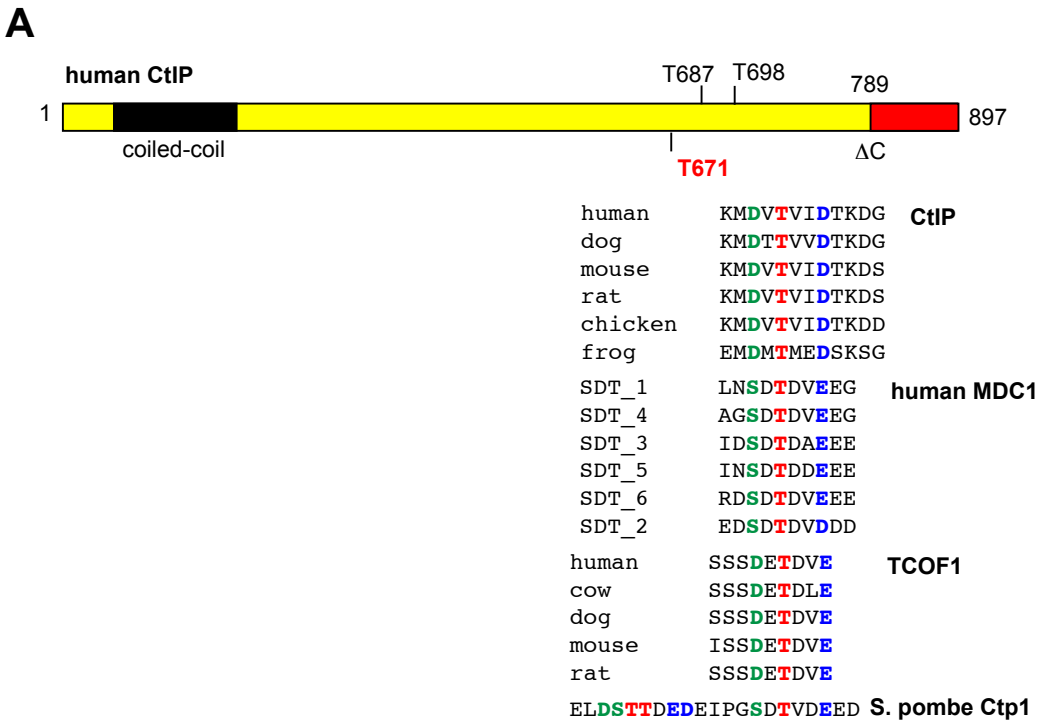



**Figure 37. CtIP and NBS1 interaction is dependent on the FHA and BRCT domains of NBS1.** 293-T cells were transiently co-transfected with Flag-CtIP (wild type) and NBS1-myc (wild type and R28A, K160M and R28A/K160M mutants, respectively). Cells were lysed and Flag-CtIP was immunoprecipitated from whole cell extracts using anti-Flag antibody beads. After washing of the beads, the immunoprecipitated proteins were analyzed by western blotting using the indicated antibodies (3 independent experiments, 1 representative experiment shown).

In order to examine if impairment in NBS1-CtIP interaction is the underlying mechanism of DNA end resection defect in FHA and BRCT domain mutants of NBS1, we sought to identify the NBS1 binding motif in CtIP. A detailed protein sequence analysis of putative CK2 sites in human CtIP revealed a cluster of potential CK2 sites towards the C-terminus (T671, T687 and T698) (Figure 38A). Sequence alignment of human CtIP with known NBS1-FHA interaction sequences revealed a "minimal consensus" region centered around T671 with an acidic amino acid (or phosphorylated Ser residue) at the -2 position relative to the central T671 and another acidic amino acid at the +3 position. To test the contribution of these putative CK2 sites towards DNA end resection, we made a deletion mutant of CtIP, lacking aa residues 650 – 750. However, unfortunately this mutant was not expressed in 293T cells indicating that it may be unstable or insoluble. We then introduced point mutations to destroy potential CK2 sites towards the C-terminus of

Flag-WT-CtIP and generated Flag-T671A-CtIP, Flag-T687A-CtIP and Flag-T698A-CtIP, respectively.

This was followed by co-transfection of the various CtIP mutants with full length NBS1myc in HEK-293T cell. As a positive control Flag-CtIP-ΔC was used. This mutant derivative of CtIP does not interact with MRN complex (Sartori et al., 2007). Flag-tag immunoprecipitations were performed and the precipitated proteins were analyzed by western blotting for the presence NBS1myc. It was observed that while WT-NBS1myc was efficiently co-immunoprecipitated with wild type and T687A and T698A mutant Flag-CtIP; it was not efficiently co-immunoprecipitated with one putative CK2 site mutant forms of Flag-T671A-CtIP (Figure 38B). This indicated that T671 could be a potential site in CtIP that mediates the interaction with NBS1. To test the role of this putative interaction site in DNA end resection, currently we are adapting a siRNA based complementation system for CtIP (Reference from Sartori lab), using the same U2OS based Flp-In T-Rex system from Invitrogen as described for NBS1 complementation.



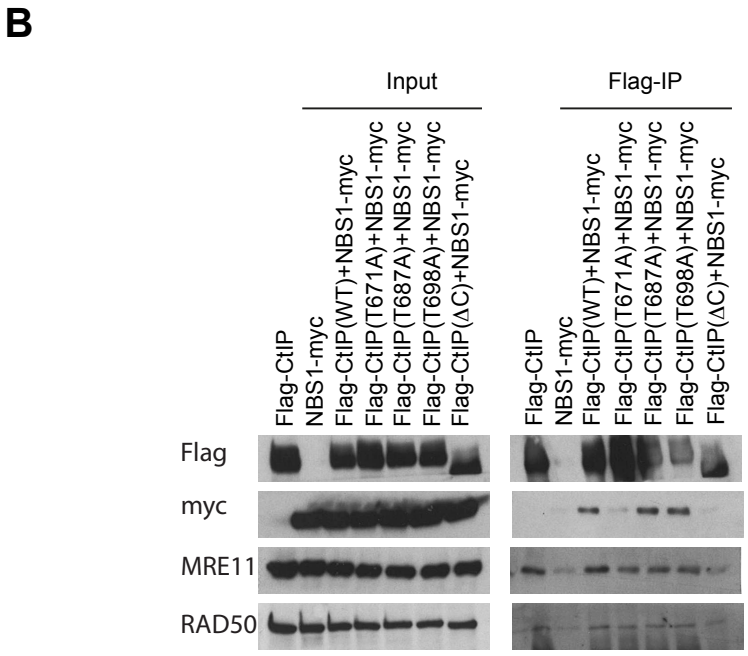




**Figure 38. (A)** Schematic representation of the primary structure of human CtIP. Shown is coiled coil region at the N-terminus and a cluster of potential CK2 sites towards the C-terminus (T671, T687 and T698). Furthermore, the red ΔC part is deleted in the C-terminal deletion mutant. Sequence alignment of T671 of human CtIP with known NBS1-FHA interaction sequences. The "minimal consensus" seems to be an acidic amino acid (or phosphorylated Ser residue) at -2 and acidic amino acid at +3. **(B)** Co-IP experiment with Flag-IP from extracts prepared from 293T cells transiently transfected with Flag-CtIP (WT, T671A, T687A, T698A and ΔC mutant) and NBS1-myc (wild type). Note that T671A and ΔC mutant CtIP are unable to efficiently interact with NBS1 (2 independent experiments, 1 representative experiment shown).

## 3.2. Investigation of the role of NBS1 in G2M checkpoint activation and checkpoint maintenance

An important aspect of the cellular responses to DNA damage induced by IR is activation of cell cycle checkpoints that delay progression of cells through the cell cycle. IR-induced checkpoints are active at the transition from the $G_1$ phase to the S phase (G1S), in the S phase (intra-S), and at the transition from the $G_2$ phase to mitosis (G2M). The activation of checkpoints facilitates maintenance of genomic integrity, by allowing time for DNA damage repair and inhibiting DNA replication or segregation into the daughter cells until DNA is completely repaired. NBS1 has been implicated as an essential mediator of cell cycle checkpoint control, after exposure to IR or radiomimetic drugs. The role of NBS1 in S-phase was initially studied in NBS cells, which exhibit radio-resistant DNA synthesis (RDS), a hallmark of intra-S-phase checkpoint failure (Kobayashi et al., 2004; Zhang et al., 2006). Later, it was shown that there are three parallel pathways to control intra-S- phase checkpoint and NBS1 has been implicated in all three of these pathways. The first branch is ATM-Chk2-CDC25A pathway (Horejsí et al., 2004; Lee and Paull, 2005), the second branch involves ATM-NBS1-SMC1 pathway (Kim et al., 2002; 2002) and the third is the ATM/FANCD2 pathway (Lim et al., 2000). In contrast, the role of NBS1 in G1S and G2M checkpoint integrity has remained controversial. While some studies reported that in NBS fibroblast cells, G1 arrest was found to be attenuated (Lee et al., 2003), others reported normal G1 checkpoint control in the same cells (Gatei et al., 2003; Girard et al., 2002). Inconsistent findings were reported, regarding the role of NBS1 in G2M checkpoint activation also. While some studies reported that NBS lymphoblastoid cells, fail to elicit G2 checkpoint activation upon IR treatment and this defect could be rescued by the ectopic expression of a wild-type NBS1 (Buscemi et al., 2001); others reported normal G2 checkpoint activation in the same cells (Antoccia et al., 1997; Girard et al., 2002).

Given the contradicting findings regarding the role of NBS1 in checkpoint activation; we sought to investigate the same in a clean genetic system, which is a novel NBS1 gene knockout in U2OS cell line. In particular, we focused on G2M checkpoint; as

majority of cancer cells arrest in G2, despite being impaired in P53 dependent G1 arrest (Löbrich and Jeggo, 2007). We examined the role of NBS1 both in G2M checkpoint activation and G2M checkpoint maintenance.

### 3.2.1. Characterization of NBS1 knockout

In order to study the role of NBS1 in G2M checkpoint activation in a clean genetic system; NBS1 gene knockout in U2OS cell line was generated, using CRISPR/Cas9-mediated gene-editing approach (Sander and Joung, 2014; Wang et al., 2014c). First, we looked into the DNA damage signaling associated phosphorylation events in NBS1 knockout cells. Wild-type and NBS1 knockout U2OS cells were exposed to 10 Gy IR and an hour after IR treatment, cells were harvested and analyzed by Western blotting for DNA damage induced phosphorylation events (Figure 40). As expected, NBS1 knockout cells were defective in phosphorylation of RPA at S4/S8. Remarkably, RPA phosphorylation at S33 was also reduced in NBS1 knockout cells, in response to IR. This is in line with a recent study that implicated NBS1 in RPA32 phosphorylation at S33 by ATR (Shiotani et al., 2014). This study also reported that RPA is gradually phosphorylated at S33 by ATR, before its phosphorylated at S4/S8 by DNA-Pkcs and indicated that RPA32 Ser33 phosphorylation could be the initial phosphorylation driven by resection. However, this study used camptothecin, which induces replication- associated DSBs that are efficiently processed by DNA end resection. Taken together, defective RPA32 S33 phosphorylation in NBS1 knockout cells implies a role of NBS1 in ATR activation, in response to IR. It is noteworthy that the role of NBS1 in ATR activation reflects a post-resection function of NBS1 and given the role of NBS1 in resection, it seems obvious that since the ATR activating structure (ssDNA) is not generated in NBS1 knockout cells, these cells are also defective in ATR activation. As previously reported for NBS cells, NBS1 knockout cells were also defective in phosphorylation of Smc1 at S966 and Chk1 at S317. Although the role of NBS1 in ATM activation is important, it is not absolutely required (Difilippantonio et al., 2007; 2005; Horejsí et al., 2004). In agreement with the previous studies, NBS1 knockout cells were only partially defective in the activation of ATM as revealed by phospho specific ATM-S1981 antibody. We observed that the effect of NBS1

depletion on ATM activation was little, but it was visible only in NBS1 knockout cells and not in the cells, in which NBS1 was depleted using siRNA. This observation emphasizes, that a complete depletion of NBS1 protein is necessary to visualize a very marginal effect of NBS1 upon ATM activation.

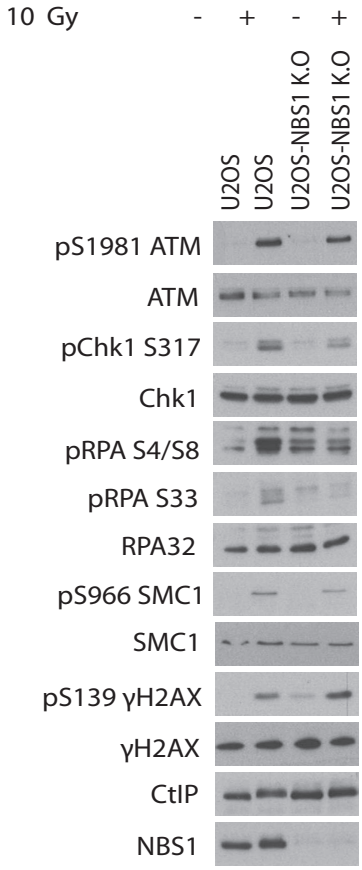



**Figure 40. DNA damage induced phosphorylation events in NBS1 Knockout.** Wild-type U2OS or NBS1 Knockout cells were exposed to 10 Gy of IR, recovered for 1hr and then lysed in SDS-lysis buffer. 50µg of the protein extracts from the indicated samples was then analyzed by western blotting using the indicated antibodies (2 independent experiments, 1 representative experiment shown).

Next, as a part of the characterization of NBS1 knockout and based on the observation that NBS1 depletion leads to a strong resection defect (see section 3.2.1), RPA foci formation in response to IR was investigated by immunostaining of cells in NBS1 knockout cells. Immunostaining and quantification of the RPA2 foci was done as described before (section 3.2.1). As seen in NBS1 knockdown cells, NBS1

knockout cells, RPA foci formation was also significantly reduced in NBS1 knockout cells (Figure 41).

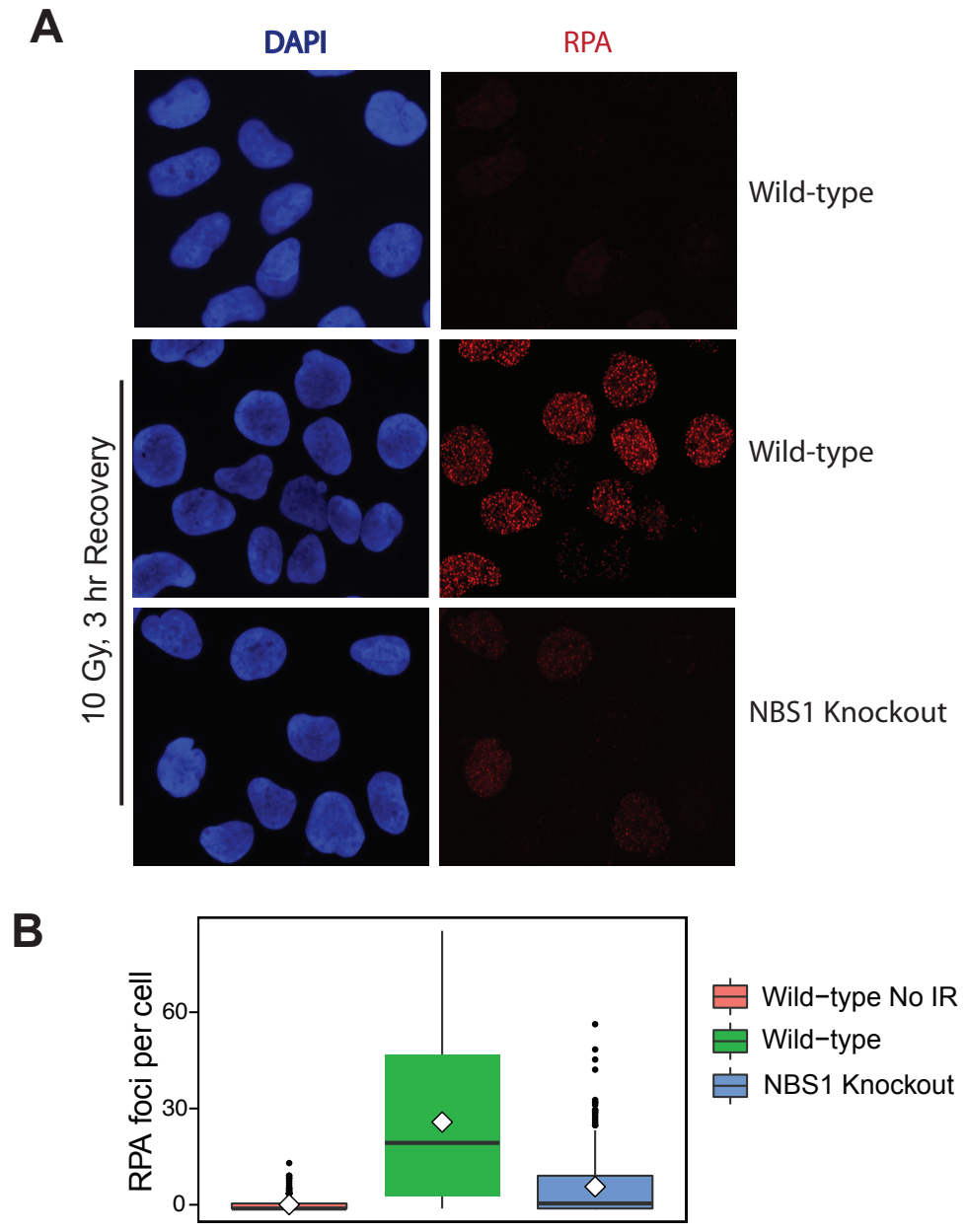



**Figure 41. Loss of RPA foci in NBS1 depleted cells. (A)** Wild-type or NBS1 knockout U2OS cells lines were seeded on coverslips. 24 h later, cells were exposed to 10 Gy of IR, recovered for 3 h fixed and immunostained using anti-RPA32 antibodies (red); nuclei were stained with DAPI (blue). **(B)** Quantification of RPA foci in NBS1 knockout cells. Around 300 cells per condition were analyzed by semi-automated image analysis (ImageJ macro). Box plots represent central 75% percentiles (box) and 95% percentiles (whiskers). Black bars indicate medians and white diamonds means of the distributions. Outliers are depicted by black dots (2 independent experiments, 1 representative experiment shown).

### 3.2.2. NBS1 is required for G2M checkpoint activation

Given the contradictory findings regarding the role of NBS1 in G2M checkpoint activation, we employed NBS1 knockout U2OS cells as a “clean” system to study the same. Many studies also reported the requirement of NBS1 In G2M checkpoint activation, but only when cells were exposed to low-doses of IR (Buscemi et al., 2001). To address if IR dose has any impact on the dependency of NBS1 in G2M checkpoint activation, a range of IR doses (0.5, 1, 2 and 4 Gy) were used to investigate the role of NBS1 in the activation of G2M checkpoint. As described before, co-staining of cells with propidium Iodide (PI) and anti-p-histone H3 Ser 10 antibodies in a FACS based assay was employed to check the cell-cycle profiles and the mitotic index. In these FACS based experiments it emerged that as previously reported, NBS1 depletion indeed interfered with the activation of G2M checkpoint and this was evident at doses as low as 1 Gy (Figure 42). Remarkably, NBS1 knockout cells were also defective in G2M checkpoint activation, at IR dose as high as 4 Gy. This was in contrast to previous studies that have implicated NBS1 in G2M checkpoint, only at low IR doses of less than 3 Gy (Girard et al., 2002). As with most of the studies on NBS1; these were also done in NBS patient derived cell lines, which retains some NBS1 function (Maser et al., 2001). Our results suggest that complete loss of NBS1 protein is important to visualize its effect on G2M checkpoint activation. This is accordance with some of the results described later, where cells depleted of NBS1 with siRNA exhibited a defect in G2M checkpoint, but only at very low IR doses (0.5 Gy).

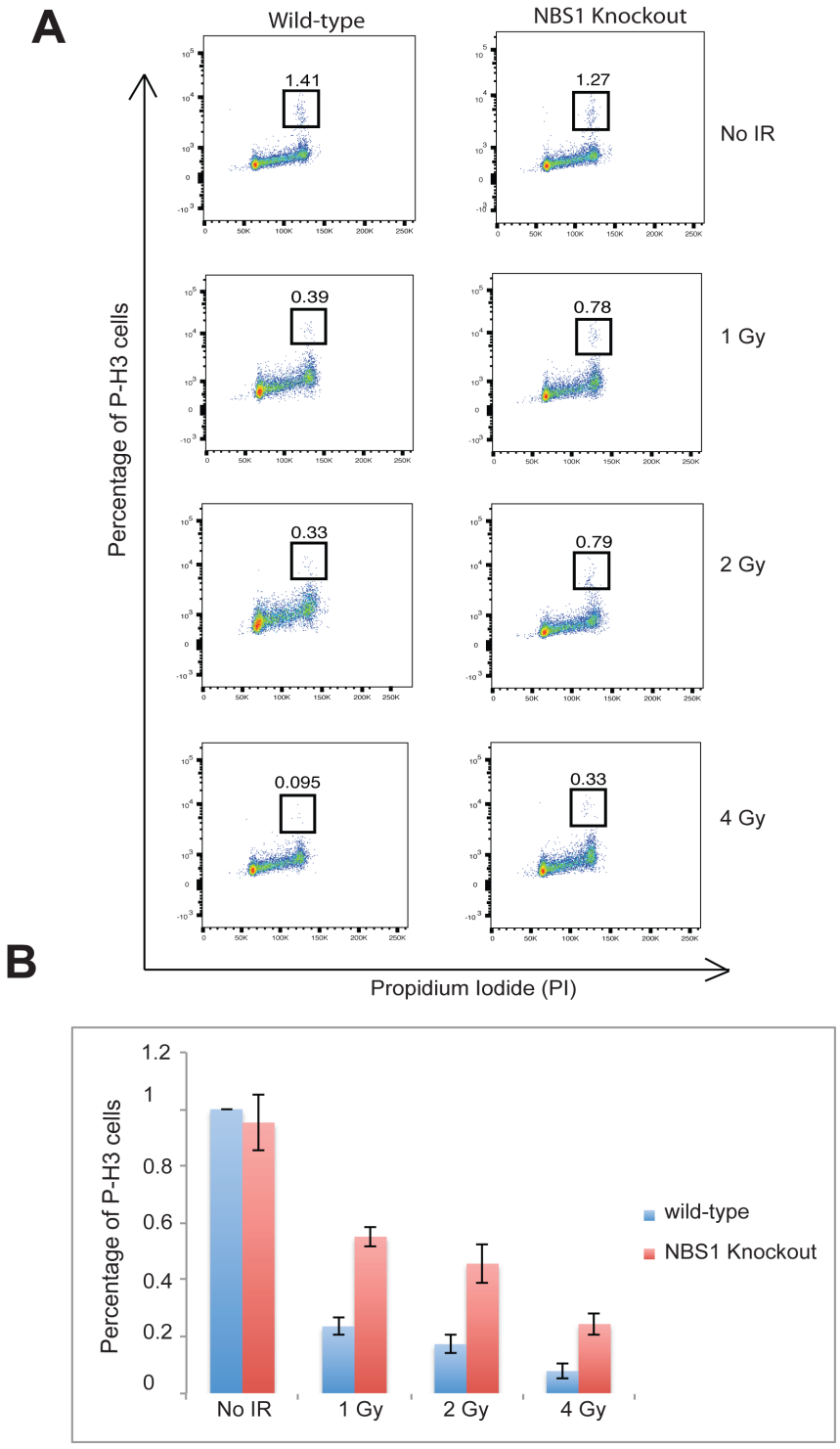



**Figure 42. NBS1 knockout cells are impaired in G2M checkpoint activation. (A)** U2OS Wild-type cells or U2OS NBS1 knockout cells were exposed to 1 Gy, 2 Gy, 3 Gy or 4 Gy of IR. Cells were harvested 1 h after IR treatment, fixed with 70% ethanol and stained with antibodies against Histone H3-Ser10p (P-H3) and propidium iodide (PI). The percentage of P-H3 positive cells was determined by flow cytometric analysis. **(B)** The bar chart represent the percentage of Histone H3-Ser10p–positive cells measured by flow cytometry. All data points represent an average of at least three replicates with error bars indicating standard error of mean.

### 3.3.3. FHA and BRCT domains of NBS1 are important for G2M checkpoint activation

Having established the role of NBS1 in G2M checkpoint activation, we next asked if the FHA and BRCT domains of NBS1 would contribute to the NBS1's role in G2M checkpoint activation. As mentioned before, NBS patient derived cells expressing the common *675del5* mutation show an impaired G2M checkpoint response at low doses of IR (less than 2Gy), but nearly normal G2/M checkpoint arrest following exposure to 3 Gy IR. This 'leaky' phenotype of NBS1 cells at higher IR doses is dependent on ATM, whose kinase activity is largely normal in these cells. This was consistent with previous findings; where NBS cells when complemented with NBS1 fragment lacking C-terminal ATM interaction motif remain impaired for IR-induced G2/M arrest (Girard et al., 2002; Stiff et al., 2008). Thus, NBS1's C-terminal dependent ATM signaling is generally considered to be the main reason for impaired G2M checkpoint activation in NBS1 cells. However, a role of FHA and BRCT domains of NBS1 has been implicated in ATR dependent but replication independent G2M checkpoint activation (Stiff et al., 2008). Since we had a functional siRNA based complementation system to study the FHA and BRCT domains of NBS1; we sought to investigate the role of FHA and BRCT domains of NBS1 in G2M checkpoint activation in this complementation system for NBS1.

To gain insight into the contribution of FHA and BRCT domains towards the overall function of NBS1 in G2M checkpoint analysis; mitotic index was quantified after irradiation, in NBS1-depleted stable cell lines that inducibly expressed WT-NBS1myc, R28A-NBS1myc (FHA mutant), K160M-NBS1myc (BRCT mutant) and D.M.-NBS1myc (combined R28A/K160M double mutant), respectively. We observed that the single FHA and BRCT domain mutants, R28A and K160M respectively were partially defective in activation of checkpoint, while the combined FHA/BRCT domain mutant (D.M.-NBS1) was largely as defective as NBS1 depleted cells in the activation of checkpoint. Strikingly and much to our expectations, from these data it also emerged that as observed in DNA end resection, the 'additive effect' in D.M.-NBS1myc cell line was prevalent in G2M checkpoint activation also. Again, as with DNA end

resection; BRCT domain mutant (K160M) was more defective in G2M checkpoint activation as compared to FHA domain mutant (R28A) and the combined FHA/BRCT domain mutant (D.M.) did not rescue the resection phenotype at all (Figure 43), emphasizing that the function of NBS1 in G2M checkpoint activation is dependent on its N-terminal phospho-interaction modules.

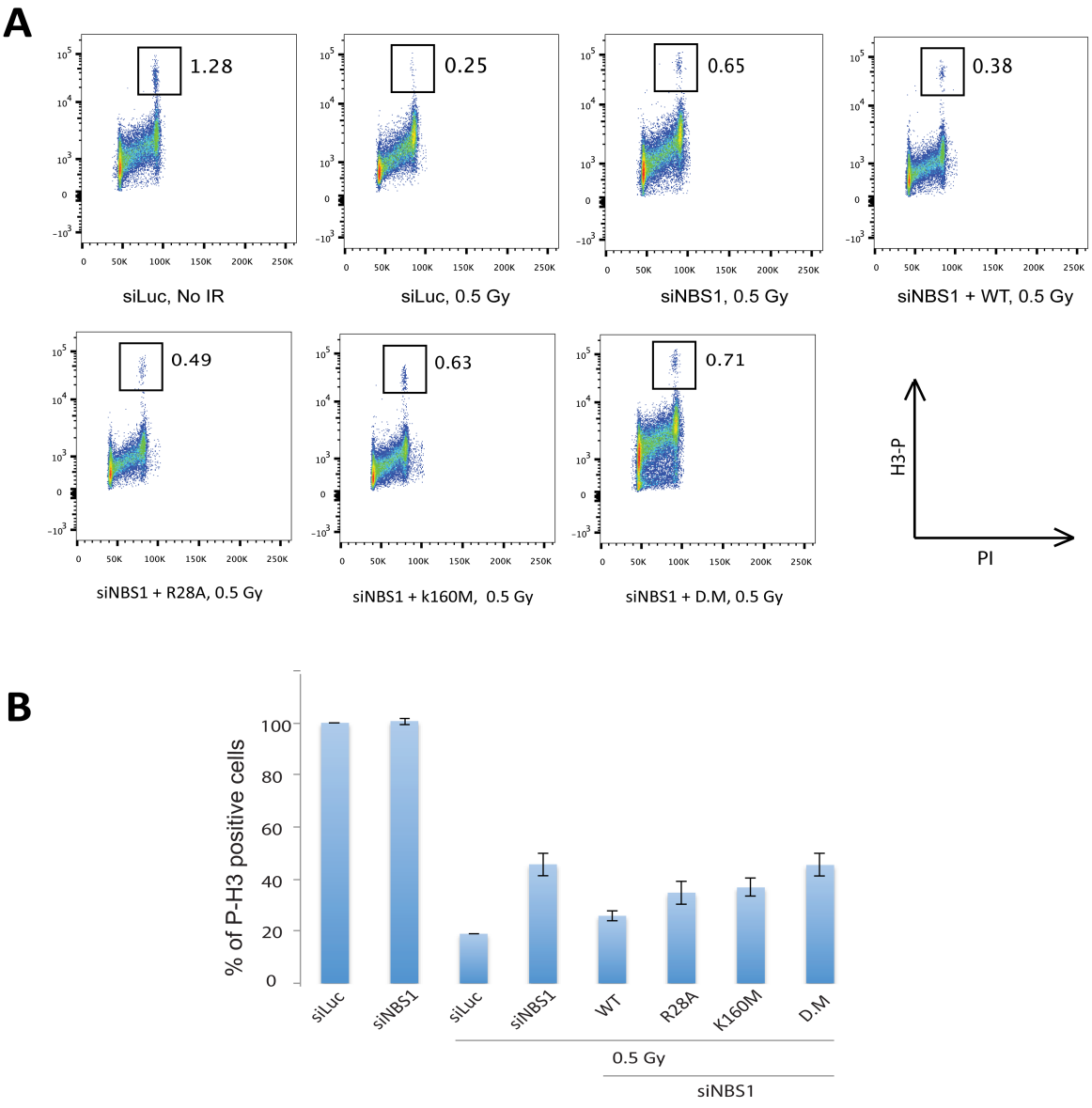



**Figure 43. Role of FHA and BRCT domains of NBS1 in G2M checkpoint activation. (A)** U2OS cells lines stably transfected with WT-NBS1myc or with R28A-NBS1myc, K160M-NBS1myc and D.M.-NBS1myc were treated with siNBS1 for 48h and then induced with doxycycline for 24h. Cells were harvested 1 h after 0.5 Gy of irradiation, fixed with 70% ethanol and stained with an antibody against phosphorylated H3 (P-H3) and propidium iodide (PI). The percentage of P-H3; positive cells was determined by flow cytometric analysis. **(B)** The graph represents three independent experiments and the error bars represent the standard error of mean.

### 3.3.4. NBS1 has no role in G2M checkpoint maintenance

DNA end resection has been linked to G2M checkpoint maintenance in a recent study, where the authors have shown that the resection dependent checkpoint pathway takes over and maintains the HR associated G2M checkpoint, by restricting mitotic entry until DNA is repaired completely (Kousholt et al., 2012). Having established the specific role of NBS1 in G2M checkpoint activation, we investigated the role of NBS1 in G2M checkpoint maintenance. To this end we first analyzed, the effect of NBS1 depletion on HR repair by U2OS based DR–GFP system, which contained a green fluorescent protein (GFP) based HR reporter construct bearing a cleavage site for the I-SceI restriction endonuclease. I-SceI expression in such cells induces a DSB that can be repaired by HR- mediated gene conversion to produce GFP-positive cells. U2OS/DR–GFP cells were treated with siNBS1 and 24h later transfected either with I-SceI expression plasmid. As expected, formation of functional GFP was impaired in siNBS1 treated cells, emphasizing the role of NBS1 in HR-mediated repair (Figure 44).

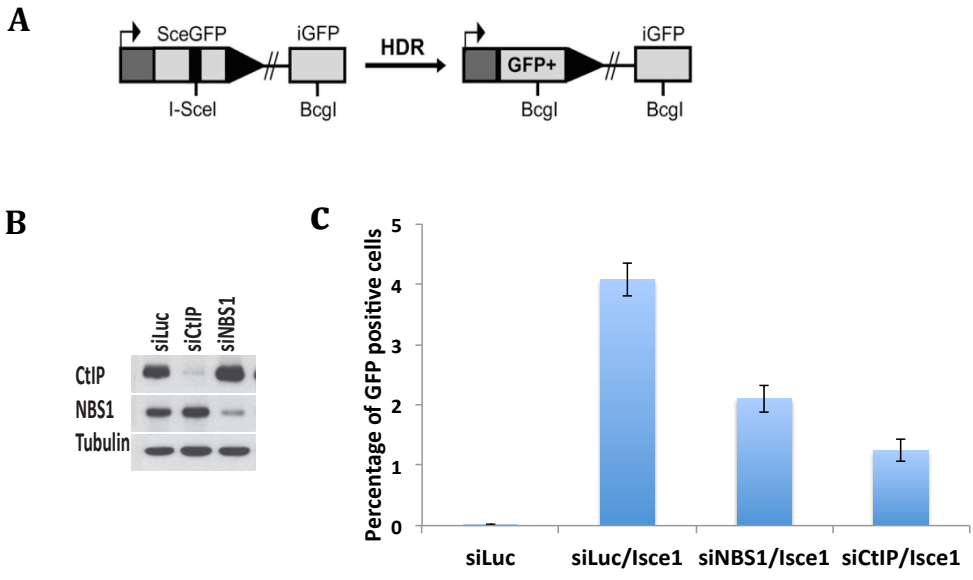



**Figure 44. NBS1 promotes HR repair. (A)** Scheme of the DR-GFP reporter for HR-mediated DSB repair. The site-specific DSB in the reporter cassette is generated by I-SceI endonuclease. Only HR events give rise to a functional GFP allele. **(B)** Western blot analysis of extracts from U2OS/DR-GFP cells transfected with indicated siRNAs. Blots were probed with indicated antibodies. **(C)** Efficiency of HR-mediated repair of I-SceI-induced DSB in U2OS/DR-GFP cells treated with indicated siRNAs. Cells were transfected with indicated siRNAs two days before transfection with I-SceI-expressing plasmid. Percentage of GFP positive

cells was measured by flow cytometry two days after DSB induction and taken as a measure of DSB repair efficiency. Values plotted represent absolute repair efficiency. All data points represent an average of at least three biological replicates with error bars indicating standard error of mean.

Next, we tested if NBS1 depletion also leads to a defect in checkpoint maintenance, as reported for the proteins involved in DNA end resection in a recent study (Kousholt et al., 2012). To analyze G2M checkpoint maintenance, cells were first exposed to IR and then trapped in mitosis by adding nocodazole, 2 h after being exposed to IR (referred as nocodazole trap experiment). The cells that exhibited a leaky G2M checkpoint were trapped in mitosis by nocodazole and a comparison of the mitotic index by FACS at two different time points, 2 h after IR and 8 h after IR (6 h after Nocodazole addition) revealed differences in checkpoint activation and checkpoint maintenance respectively. Strikingly, NBS1 knockout cells exhibited a very little defect when compared to CtIP depleted cells in the maintenance of G2M checkpoint in this assay. Moreover, NBS1 knockout cells depleted of CtIP exhibited very little affect, when compared to wild-type cells depleted of CtIP in checkpoint maintenance (Figure 45). These findings suggest that the effect of CtIP depletion on checkpoint maintenance is counteracted in the cells that don't express NBS1. These results were totally counterintuitive, given the positive role of both NBS1 and CtIP in DNA end resection. To get a deeper insight into this, we treated U2OS NBS1 knockout cells with IR and estimated checkpoint activation and maintenance by blotting for Chk1 phosphorylation. As reported for CtIP (Kousholt et al., 2012), Chk1 phosphorylation as measured by pS317 Chk1 and pS345 Chk1 antibodies, revealed that Chk1 was phosphorylated as soon as 15 minutes after IR in NBS1 knockout cells. In contrast the levels of phosphorylated Chk1 was substantially reduced at 2-3 h after IR, when compared to Wild-type cells (Figure 46). In summary, the role of NBS1 in maintenance of Chk1 phosphorylation was revealed in the western blot analysis, but role of NBS1 in checkpoint maintenance was not discernible when NBS1 knockout cells defective in G2M checkpoint maintenance were trapped in mitosis by nocodazole.

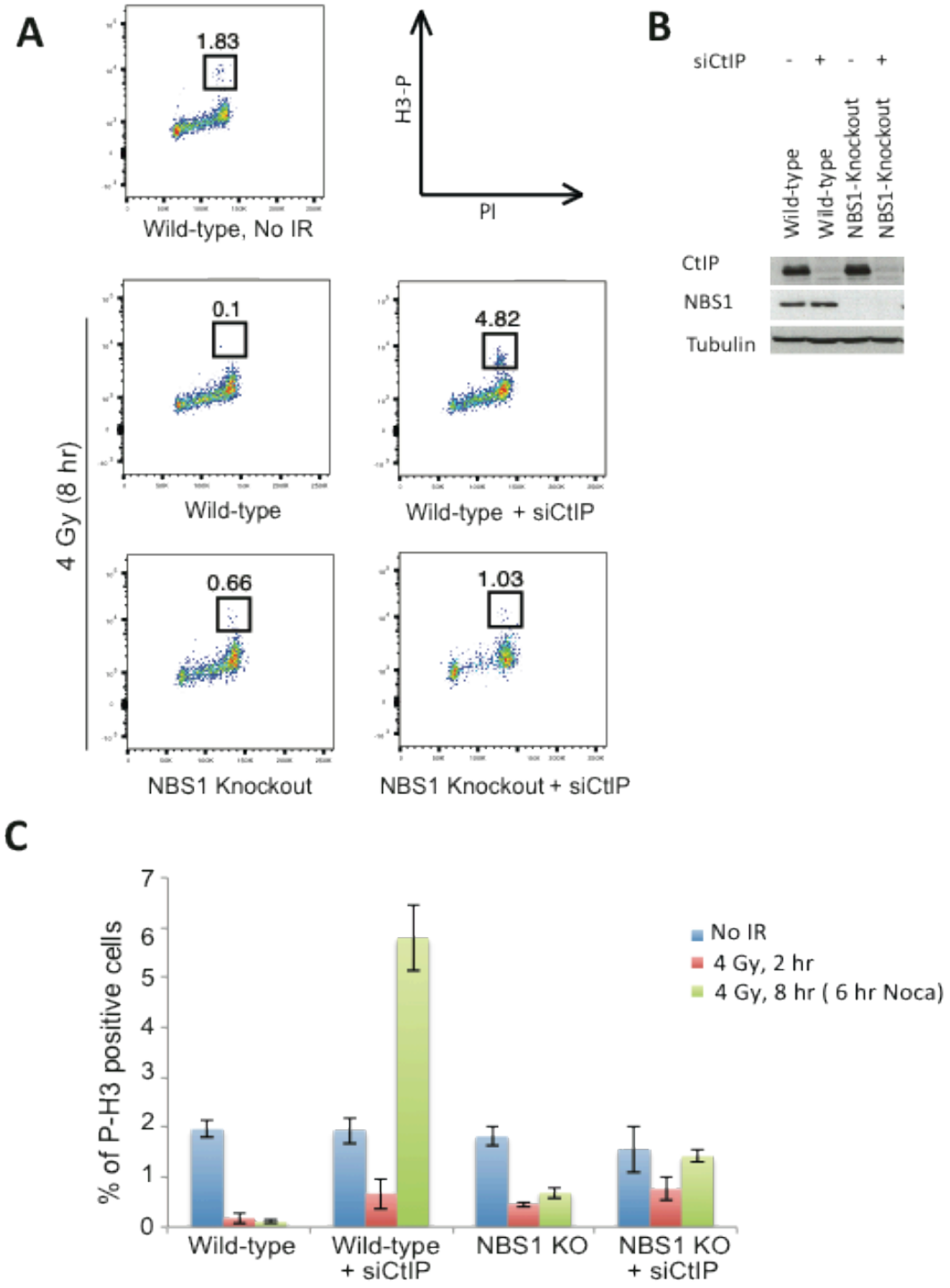



**Figure 45. NBS1 knockout cells exhibit different response when compared to CtIP depleted cells in IR-induced checkpoint maintenance.** (A) U2OS Wild-type cells or U2OS NBS1 knockout cells (NBS1 KO) were transfected with the siCtIP for 72 h and samples were fixed at the indicated time points after IR. Nocodazole was added 2 h after IR. The bar chart represents the percentage of phosphorylated Histone H3-Ser10p (P-H3) positive cells measured by flow cytometry. All data points represent an average of at least three replicates with error bars indicating standard error of mean.

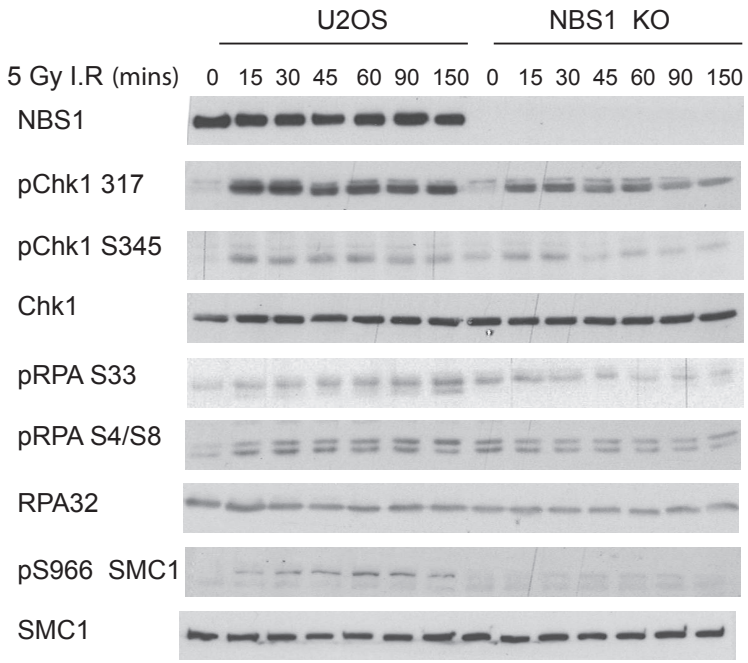



**Figure 46. NBS1 is required for sustained checkpoint signaling.** U2OS Wild-type or U2OS NBS1 Knockout cells were exposed to 5 Gy of IR, recovered for indicated time points and then lysed in SDS-lysis buffer. 50 µg of the protein extracts from the indicated samples was then analyzed by western blotting using the indicated antibodies (2 independent experiments, 1 representative experiment shown).

To move further, it was important to understand why NBS1 knockout cells exhibit unrestrained checkpoint maintenance response in nocodazole trap experiments, despite being defective in DNA end resection. We considered the possibility that may be checkpoint maintenance defect in NBS1 knockout cells is not evident in nocodazole trap experiments because these cells accumulate in G2 upon IR treatment. G2 accumulation is ATM independent (ATR dependent) and it is actually enhanced by the lack of ATM, as well as NBS1 (Xu et al., 2002). Given the role of NBS1 in intra-S phase checkpoint, we reasoned that may be NBS1 depleted cells don't exhibit heightened mitotic Index in nocodazole trap experiments because they fail to arrest in S phase in response to the irradiation and later exhibit enhanced G2 accumulation. Should this be true, one would expect that the cells that get trapped in mitosis and lead to heightened mitotic index in CtIP depleted cells were in S-phase at the time of irradiation. To test this possibility, cells were labeled with EdU before performing FACS based Nocodazole

trap experiments. Remarkably, we observed that the huge mitotic population of CtIP depleted cells was not coming from S-phase of the cell cycle as all these cells were negative for EdU (Figure 47). This implies, that the unrestrained checkpoint maintenance manifested in the form of much lower mitotic index in NBS1 knockout cells compared to CtIP depleted cells, could not be the result of any S-phase checkpoint anomalies that could have resulted in enhanced G2 accumulation of cells in NBS1 knockout cells. Taken together it can be hypothesized that NBS1, is not required for checkpoint maintenance.

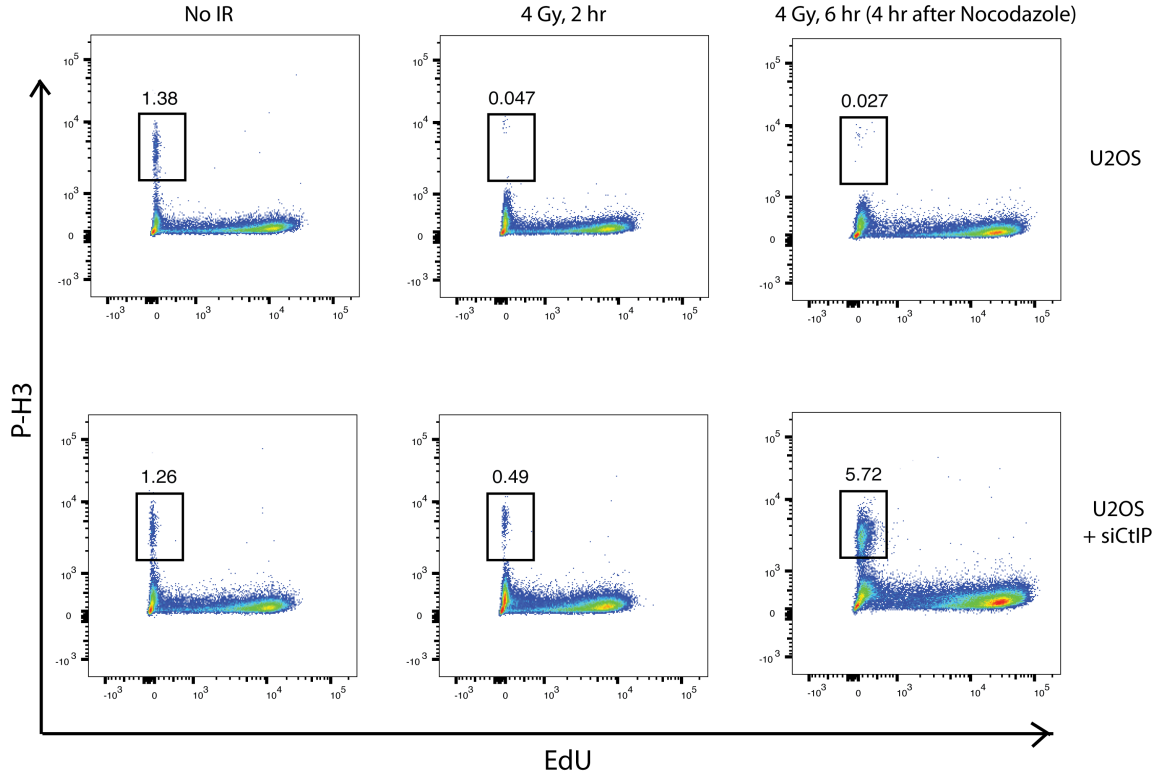



**Figure 47. The huge mitotic population of CtIP depleted cells comes from G2 phase.** U2OS were left transfected with siLuc or with siCtIP for 65 h. Cells were then pulse-labeled with 10 μM EdU for 30 min before treating them with X-Ray dose of 4 Gy. After 2 h of X-Ray exposure, Nocodazole was added to the cells and finally cells were fixed 6 h after IR exposure. The samples were then permeabilized and stained with antibodies against Histone H3-Ser10p (P-H3). EdU detection reaction was carried out at this point, followed by DAPI staining. The black square marks the gate, indicating the percentage of p-H3 positive cells and EdU negative cells. (2 indepenent experiments, 1 representative experiment shown).

## 4. Discussion

NBS1 is the most enigmatic component of the MRN complex. Functionally, it is mainly considered to act as a regulatory subunit of MRN complex. It is also a tumor suppressor protein and hypomorphic mutations in NBS1 are associated with a rare genetic disorder characterized by microcephaly, immunodeficiency, and cancer predisposition. In higher eukaryotes NBS1 is essential for radioresistance and sister chromatid exchange, suggesting an involvement of NBS1 in HR repair (Sakamoto et al., 2007; Tauchi et al., 2002). Since NBS1 is required for HR and given the fact that HR is mainly regulated at the level of DNA end resection, we thought it would be interesting to investigate the role of NBS1 in DNA end resection. In addition, this function of NBS1 has not yet been extensively studied in mammalian cells. In the first part of this work, we generated a novel cellular complementation system for NBS1 in U2OS cell lines, using the U2OS Flp-In T-REx system. In the next phase of this study, we employed this complementation system to investigate the function of NBS1 in DDR and discovered that NBS1 plays an important role in DNA end resection and in the G2M checkpoint activation, which is dependent on its FHA and BRCT domains. Remarkably, we found that the FHA and BRCT domains contribute to the overall function of NBS1 in an additive manner.

### 4.1. siRNA based complementation system for NBS1

Most of the structure/function studies done to investigate the physiological role of NBS1 and to understand the mechanisms by which this protein is implicated in the DDR were performed in cell lines derived from NBS patients, harboring the most common *657del5* mutation in NBS1. However, *657del5* is a hypomorphic mutation that determines synthesis of two truncated protein fragments p26 and p70 of NBS1, albeit in very low levels (Maser et al., 2001). While several NBS patient cell lines have been isolated, one of the most frequently used cell lines is NBS1-iLB. This cell line was derived from fibroblasts of a Polish patient harboring the most common NBS1 mutant form *675del5* (Kraakman-van der Zwet et al., 1999; Maser et al., 2001). It

was then subsequently immortalized by SV40 transformation which gave rise to the NBS1-iLB cell line that up until today is still the most frequently used cell line for NBS1 structure/function analysis in human cells. Both primary and immortalized NBS1-iLB cell lines display the same cellular characteristics and hence were accepted as a good model to study NBS1. However, NBS1-iLB synthesize detectable levels of p70 fragment of NBS1 (Krüger et al., 2007; Lee et al., 2003) and because p70 can form a complex with MRE11, it represents a partially functional form of NBS1 (Maser et al., 2001). The partial functionality of p70 fragment of NBS1 is also reflected by the fact that expression of the *657del5* human *NBS* allele rescues the lethality of *Nbs1*$^{-/-}$ mice mutant (Difilippantonio et al., 2005). Several murine models to study NBS1 were also developed by targeted disruption of the exons at the 5' end (Kang et al., 2002; Williams et al., 2002). However, these mice also expressed low levels of C-terminal NBS1 protein and didn't phenocopy all the manifestations of NBS. Efforts to generate NBS1 null mutants in mice largely failed, because null mutation of NBS1 is embryonic lethal (Zhu et al., 2001) In addition, a humanized mouse model was developed in which the human NBS1 gene replaced the mouse NBS1 gene (Difilippantonio et al. Nat Cell Biol., 2005; Difilippantionio et al. J. Exp. Med. 2007). Some studies also applied Cre-loxP technology to conditionally knock out NBS1 in certain tissues in order to overcome the embryonic lethality of NBS1 deletion (Demuth et al., 2004; Frappart et al., 2005; Reina-San-Martin et al., 2005).

All of the structure/function studies on NBS1 and its FHA and BRCT domains were done by functional complementation of either human immortalized NBS patient-derived cell lines or by functional complementation of conditional mouse NBS1 knockout models with human NBS1. Both systems are not ideal because in the case of human NBS patient-derived cell lines, functional studies on NBS1 mutants basically means studying one hypomorphic mutation in the genetic background of another hypomorphic mutation, which is prone to artifacts. In the mouse system loss of mouse NBS1 was compensated for with expression of human NBS1. This is also not ideal because NBS1 is the least conserved subunit of the MRN complex and there are significant differences between the mouse and the human NBS1 sequence. To perform structure/function analysis focusing on the FHA and BRCT domains of NBS1,

we generated a new NBS1 complementation system based on siRNA, which allowed us to express FHA and BRCT domain mutants of full-length NBS1 in cells that do not express detectable levels of endogenous NBS1. We utilized the osteosarcoma cell line U2OS for the generation of our complementation system because this cell line is widely used in the DDR field and thus, we were able to directly compare our data to other published studies using the U2OS cell line.

The rationale behind generating a siRNA based complementation system was based on the long-standing technical difficulty to generate somatic knockouts in human cell lines. However, in the final stages of this project, with the arrival of the new easy-to-use and highly efficient CRISPR/Cas9 genome editing technology, we could also generate NBS1 gene knockouts in U2OS, where all the three U2OS NBS1 alleles were inactivated by insertion/deletion mutations that cause frameshifts and nonsense mutations. This indicated that NBS1 null mutations are not lethal in human cancer cell lines and that structure/function analysis of NBS1 could also be done in a real genetic null background in U2OS cells in the future. In the case of this study, we could use the NBS1 knockout U2OS cell lines to rigorously test and validate our siRNA based complementation system. As we consistently observed the same phenotypes in the NBS1 knockout U2OS cells as compared to the cells in which NBS1 was depleted by siRNA, we concluded that the data achieved with our siRNA-based complementation system were based upon solid grounds.

In our genetically engineered U2OS cell lines the expression of ectopic NBS1 upon induction was higher as compared to endogenous NBS1 expression levels. However, overexpression of recombinant NBS1 was not toxic for the cells and we observed complete functional complementation with the wild type NBS1 expressing cell line, thus indicating that NBS1 expression levels are not a critical parameter in U2OS cells. We also observed low levels of recombinant NBS1 expression in the absence of induction by DOX in all of the cell lines, but this did not seem to be influencing cell growth and cell survival. The control and recombinant NBS1-expressing cell lines exhibited similar growth rates and similar cell cycle profiles, ruling out cell cycle artifacts or artifacts caused by NBS1 overexpression. Finally, we also showed that the

recombinant NBS1 is readily incorporated in the MRN complex indicating that functional complementation takes place in the context of the intact MRN complex. In summary, our siRNA based complementation system is clearly superior to the NBS1-iLB-based complementation system, as it is a genetically “clean” system. In addition, our system may also be superior to the humanized mouse complementation system because we express recombinant human NBS1 in a human cellular background as compared to human NBS1 in a mouse cellular background.

### 4.2. Role of NBS1 in DNA end resection

The precise regulation of DSB repair is crucial for the maintenance of genomic stability. DSB repair by HR is limited to S/G2 phases of the cell cycle when the intact sister chromatid provides the template for homology directed repair. DNA-end resection is the most critical regulatory step that commits the repair to HR. The MRN complex is essential for the initiation of DNA-end resection, and its function in this process is mainly attributed to the nuclease activity of MRE11. A contribution of NBS1 in the generation of ssDNA at sites of DSBs has been observed before, mainly in the form of defective RPA foci formation in its absence, (Chen et al., 2008; Falck et al., 2012; Jazayeri et al., 2006) but its regulatory role in DNA end resection has remained elusive so far. In this study, we showed by different methods that NBS1 plays an important role in DNA end resection. We first used RPA2 foci formation as a marker for ssDNA generation at sites of DSBs (Raderschall et al., 1999; Sartori et al., 2007) and observed that NBS1 depletion severely impairs RPA foci formation. We then substantiated our findings, using a phospho-specific anti-RPA2 pS4/S8 antibody, a widely used surrogate marker for ssDNA generated by DNA-end resection (Murina et al., 2014; Sartori et al., 2007). Because RPA and pRPA foci formation indicate RPA binding to stretches of ssDNA, they are not direct markers for the generation of ssDNA at sites of DSBs by DNA end resection. Thus, we employed an anti-BrdU antibody staining technique that detects BrdU incorporated in DNA, when DNA is in single-stranded form (Bunting et al., 2012; Sartori et al., 2007). Analysis of DNA end resection by BrdU antibodies revealed that as with RPA and pRPA staining, NBS1

depleted cells were defective in ssDNA generation at sites of DSBs. Collectively, our results revealed that NBS1 plays an essential role in DNA end resection and that the resection phenotype of NBS1 depleted cells is almost as strong as the one elicited by CtIP depletion.

An important observation of this project was that DNA end resection is independent of MDC1. This also indicated that the formation of MRN foci is not important for DNA end resection, as MRN foci formation depends on the interaction between NBS1 and MDC1 (Chapman and Jackson, 2008; Lukas et al., 2004a; Melander et al., 2008; Spycher et al., 2008). Based on these findings, we concluded that the recruitment mechanism of the MRN complex to microscopically discernible foci at ssDNA compartments is distinct from its recruitment mechanism in chromatin regions flanking DSBs, which is mediated by MDC1. For NBS1, it has been reported that it is capable of binding to the ssDNA compartment in an MDC1 independent manner (Lukas et al., 2004a). Because neither MDC1 nor MDC1-dependent chromatin association of the MRN complex are required for DNA end resection, it seems likely that the MDC1 independent recruitment of a small fraction of NBS1 in the form of “microfoci”, represents the site of active DNA end resection. CtIP is not required for the recruitment of NBS1 to DSB-flanking chromatin (You et al., 2009) and it is generally accepted that CtIP is not recruited to DSB-flanking chromatin but its DSB recruitment is confined to the ssDNA compartment (Chen et al., 2008; Yuan and Chen, 2010). However, it still remains unknown if the MRN complex and CtIP are independently recruited to the ssDNA compartment at sites of DSBs. Although it would be interesting to investigate if NBS1 or CtIP can influence each other’s binding to ssDNA, the interpretation of such an analysis would be very difficult. This is mainly because both in absence of CtIP or NBS1, resection is impaired and hence there is no or very low ssDNA generation at sites of DSBs. Therefore we would expect to see defective CtIP foci formation in the absence of NBS1 and vice versa. However, whether or not this is caused by defective resection or by a more direct role of CtIP and NBS1 in the regulation of protein recruitment at sites of DSBs and foci formation would still be elusive.

We also observed that the cells depleted of MDC1 formed brighter RPA foci and also exhibited more phosphorylation of RPA at S4/S8 when compared to control cells (Figure 31). At the moment we do not understand this phenomenon but we hypothesize that this observation could be explained by MDC1's role in the maintenance of 53BP1 at sites of DSBs (Stewart et al., 2003). In the G1 phase of the cell cycle, 53BP1 and its cofactor RIF1 block DNA end resection, while in S and G2 phase exclusion of 53BP1 from chromatin surrounding DSBs leads to extensive end resection (Bouwman et al., 2010; Bunting et al., 2010; Callen et al., 2013; Chapman et al., 2012a; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013). Based on these studies, it seems likely that in the absence of MDC1, the physical impediment of DNA end resection poised by 53BP1 is lost and this leads to the observed hyperresection phenotype.

Another important finding of this study is that the function of NBS1 in DNA end resection is dependent on its FHA and BRCT domains. Three different resection based markers - RPA, pRPA and BrdU in immunofluorescence microscopy revealed that the FHA and BRCT domains of NBS1 exhibit an "additive effect" on DSB end resection (Figure 25, Figure 26, Figure 27). The single FHA and BRCT domain mutants were partially defective in resection, while the combined FHA/BRCT domain mutant was as defective as NBS1 depleted cells in the generation of ssDNA at sites of DSBs. Remarkably, the BRCT domain mutant was more defective in resection than the FHA domain mutant in all the assays. A flow cytometry-based method was also adopted (Forment et al., 2012) to assess the role of NBS1 and its FHA/BRCT domains in DNA end resection, which also revealed that the FHA and BRCT domains of NBS1 are indispensible for DNA end resection. Apart from the FHA and BRCT dependent interactions of NBS1 with MDC1, the recruitment of MRN complex to sites of DNA damage is also dependent on poly-ADP-ribose polymerases (PARPs), that load poly-ADP ribose (PAR) at the sites of DNA damage (Bryant et al., 2009; Haince et al., 2008). Interactions between the MRN complex and PAR have previously been attributed to the MRE11 subunit of MRN complex, but recently the BRCT domains of NBS1 were also implicated in PAR binding. In a screen for FHA and BRCT domain interactions with PAR the BRCT domains of NBS1 were identified as efficient PAR

binding modules (Li and Yu, 2013) . In this study, the DNA damage induced localization of the MRN complex to DNA damage sites was delayed in the NBS1-BRCT1 domain mutant (K160A), which was also impaired in binding to PAR. However, these findings are not in agreement with previous studies where it was shown that both that FHA domain and the BRCT domains of NBS1 are important for both the early and late recruitment of the MRN complex to damage sites (Hari et al., 2010; Lukas et al., 2004b). Nevertheless, it may be possible that at an early time point, interactions between the NBS1-BRCT domain and PAR could be implicated in the early MDC1 independent recruitment of the MRN complex to sites of DNA damage. In line with these findings, we observed that the BRCT domain mutant of NBS1 (K160M) is more impaired in DNA end resection when compared to FHA domain mutant.

The FHA domain of NBS1 was also recently proposed to interact with the alternative clamp loader Rad17 and this interaction was suggested to promote MRN complex retention at sites of DSBs. A direct interaction between the C-terminus of Rad17 and the NBS1-FHA domain was implicated in the retention of the MRN complex at breaks (Wang et al., 2014b). It was also reported in the same study that Rad17 depletion resulted in a decrease of RPA as well as of pRPA (Ser4/8) and BrdU foci-positive cells, implicating the function of Rad17 in DNA end resection. However, when we depleted cells of Rad17 and examined DNA end resection by examining RPA phosphorylation at S4/S8, RPA or pRPA (S4/S8) foci-positive cells upon IR exposure, we observed no effect of Rad17 depletion on DNA end resection.

CDKs are emerging as important regulators of the DNA damage response and they promote DNA end resection by phosphorylating key proteins of DNA end resection such as CtIP (Huertas and Jackson, 2009) and EXO1 (Tomimatsu et al., 2014; Zhu et al., 2001). Two studies recently reported a cell cycle regulated phosphorylation of NBS1 at S432 residue. One of these studies implicated CDK dependent NBS1 phosphorylation at S432 residue in DNA end resection during the S and G2 phases of the cell cycle (Falck et al., 2012). In contrast, another study reported normal DNA end resection in the absence of NBS1-S432 phosphorylation (Wohlbold et al., 2012).

In order to resolve this discrepancy we decided to test the role of NBS1 phosphorylation at S432 in DNA end resection and thus, we generated a cell line that stably expressed S342A-NBS1myc in an inducible manner, using our U2OS Flp-In T-REx system. Expression of siNBS1 resistant S432A-NBS1myc fully rescued the resection defect observed in U2OS cells depleted of endogenous NBS1, as tested by BrdU, pRPA and RPA foci formation. These results, together with the study by Wohlbold et al. 2012 thus indicate that NBS1 phopshorylation at S432 is not essential for DNA end resection and may only be required in the genetic background of the NBS-iLB1 cell line, which was used in the study by Falck et al., 2012.

In a further quest to find a possible mechanistic explanation of the effect of FHA and BRCT domains mutations of NBS1 in DNA end resection, we tried to find a possible DNA end resection associated interaction partner of NBS1. Many studies have reported that CtIP interacts with the MRN complex, although the mechanism of this interaction is not clear. The collaborative action of CtIP and the MRN complex in DNA end resection has been reported in many studies (Huertas and Jackson, 2009; Murina et al., 2014; Sartori et al., 2007). A recent study showed that mutations in the FHA/BRCT region of NBS1 impairs DNA repair by HR (Wang et al. 2013). This study reported novel CDK sites in CtIP, which mediate associations with the FHA/BRCT domains of NBS1, facilitating phosphorylation of CtIP by ATM at T859 and recruitment of BLM1 and EXO1 to sites of damage. Another study however, reported that CtIP is phosphorylated at T818 by ATR and phosphorylation of CtIP at T818 promotes stable association of CtIP to damaged chromosomes, allowing CtIP-dependent resection (Peterson et al., 2013). Importantly, NBS1 binds via its FHA and BRCT domains to a very specific phosphorylated consensus motif called “SDT motif”, where a Ser and a Thr residue are embedded in an acidic sequence environment and are constitutively phosphorylated by acidophilic kinases such as Caseine kinase 2. This was observed in MDC1 (Chapman and Jackson, 2008; Melander et al., 2008; Spycher et al., 2008)) and TCOF1 (Larsen et al., 2014) in human cells, as well as in in the yeast *S. cerevisiae* where the FHA domain of Xrs2 (the *S. cerevisiae* orthologue of NBS1) interacts with the XRCC4, orthologue Lif1 via phosphorylated SDT-like motifs (Matsuzaki et al., 2012). The fission yeast CtIP orthologue, Ctp1 contains similar SDT

motifs that are important for its interaction with the FHA domain of *S. pombe* Nbs1, which is important for DNA damage resistance (Lloyd et al., 2009; Williams et al., 2009) In light of all these studies, we speculated that the FHA/BRCT region of NBS1 would interact with SDT motifs present in a DNA end resection associated factor such as CtIP, rather than the CDK motifs that were suggested by Wang et al (Wang et al., 2013b). Remarkably, a detailed protein sequence analysis and sequence alignment of human CtIP revealed a cluster of potential CK2 “minimal consensus" sites towards the C-terminus (Figure 38A). These sites resemble the known SDT motifs and thus can potentially interact with NBS1 in human cells. Co-immunoprecipitation experiments revealed that T671 could be a potential site in CtIP that mediates the interaction with NBS1 (Figure 38B). We are thus currently generating cell lines that inducibly express CtIP mutants in which all the potential SDT-like motifs are altered. DNA end resection analysis in cell lines that express these phospho-mutant forms of CtIP in the absence of endogenous CtIP will eventually clarify whether or not these putative CK2 sites in CtIP are functionally important. If they are, we will continue to analyze these sites and investigate if they are phosphorylated in vivo and if they are indeed forming an evolutionary conserved NBS1 interaction region in CtIP.

It could be very well possible that the NBS1 and CtIP interaction is not the only association that links MRN complex to CtIP. Some previous work in the lab has elucidated that endogenous CtIP is pulled down with MRN complex in reconstituted NBS1-iLB1 patient cell lines. In contrast, no CtIP was co-immunoprecipitated with MRN complex, harboring the FHA and BRCT domain mutants of NBS1. Surprisingly, even in non-complemented NBS1-iLB1 cells, CtIP co-immunoprecipitated with MR complex, suggesting that there could be multiple point of interaction between CtIP and MRN complex. This was in agreement with previous reports that CtIP interacts with all three components of the MRN complex *in vitro* (Sartori et al., 2007; Yuan and Chen, 2010) and although NBS1 can directly interact with CtIP *in vitro*, the *in vivo* interaction of NBS1 with CtIP is significantly increased when NBS1 forms a complex with MRE11 and RAD50 (Chen et al., 2008). In agreement with all these results, we also observed that the interaction between NBS1 and CtIP is strongest between full

lengths WT-NBS1 and CtIP. The CtIP variant that lacks MRE11 interaction sites (CtIPΔC), interacts much weakly compared to full length CtIP with NBS1, suggesting that as many studies have hinted, multiple points of interaction between CtIP and individual subunits of MRN complex could lead to a overall stable interaction between CtIP and MRN complex.

Collectively, our data indicate that NBS1 has an additional role distinct from the localization of MRN complex to the nucleus in DNA end resection, which is dependent on the FHA and BRCT domains of NBS1. Although it is still not clear how exactly the FHA and BRCT domains of NBS1 are mechanistically implicated in DNA resection, preliminary results suggest that they may mediate a direct interaction with the key factor of resection, CtIP. Future studies should clarify if putative CK2 sites in human CtIP that mediate the interaction of CtIP with NBS1 (Figure 38B) has any functional implication in DSB repair, DNA end resection, and cell survival.

### 4.3. Role of NBS1 in G2M checkpoint activation and maintenance

One of the hallmarks of NBS1 deficient cells is defective cell cycle checkpoint activation. Early studies mainly focused on NBS1' s role in the intra-S phase checkpoint. However, several lines of evidence also suggested its implication in the G2M checkpoint. In this study we mainly focused on a potential role of NBS1 in G2M checkpoint maintenance, because G2M checkpoint maintenance has recently been linked to DNA end resection (Kousholt et al. 2012). In agreement with most of the studies that addressed the role of NBS1 in cell cycle checkpoint activation, we observed that NBS1 deficient cells are defective in G2M checkpoint activation. Interestingly though, our data indicate that complete silencing of NBS1 expression is essential to yield a significant G2M checkpoint defect. Such complete silencing cannot be achieved by siRNA transfection but only by a complete knock out of the NBS1 gene. Hence, in cells depleted from NSB1 by siRNA we observed a significant defect in G2M checkpoint activation only upon exposure to low doses of irradiation (Figure 43). This was in contrast to NBS1 knockout cells, which exhibited impaired

G2M checkpoint activation both at low and high IR doses (Figure 42), suggesting that the complete removal of NBS1 protein is critical to yield a significant G2M checkpoint activation defect at higher doses of radiation.

A recent study (Kousholt et al., 2012) proposed that DNA end resection is crucial for the maintenance of the G2M checkpoint. This was based on the observation that CtIP-depleted cells were not significantly impaired in the initial activation of the G2M checkpoint but that they were mainly defective in the G2M checkpoint maintenance. The authors of this study concluded that CtIP mediated resection was required for G2M checkpoint maintenance. This interpretation was further supported by the observation that EXO1 and BLM depletion that also resulted in reduced resection, also showed premature entry into mitosis after induction of DNA damage (Kousholt et al., 2012). Since our data showed that NBS1 is crucial for DNA end resection, we considered that its depletion would also yield a G2M checkpoint maintenance defect, similar to the one induced by CtIP depletion. Surprisingly though, we did not observe any effect of NBS1 depletion on G2M checkpoint maintenance (Figure 45). This was quite unexpected, given the observation that similar to CtIP depletion, NBS1 depletion also impairs Chk1 activation at later time points (Figure 46).

Since NBS1 depletion has a slightly less pronounced effect on DNA end resection when compared to CtIP depletion (Figure 25, 26, 27), we considered the possibility that the residual resection activity upon NBS1 depletion may be sufficient to maintain the G2M checkpoint. To test this possibility, we depleted CtIP by siRNA in NBS1 knock out cells. Surprisingly, these cells were still largely proficient in the maintenance of the G2M checkpoint, even though CtIP depletion in a NBS1 wild type background led to a dramatic G2M checkpoint maintenance defect. These observations have several implications: first, they suggest that the G2M checkpoint maintenance defect observed in CtIP depleted cells is not primarily caused by the absence of DNA end resection. Second, they also suggest that NBS1 loss is dominant over CtIP loss in the context of G2M checkpoint maintenance. While CtIP down regulation leads to a G2M checkpoint maintenance defect in the presence of NBS1, it does not in its absence. While we do not yet have an explanation for this surprising

observation, we currently think that some other function of CtIP; independent of its function in DNA end resection is critical for the maintenance of G2M checkpoint.

In summary, our data suggests that NBS1 has an important function in the activation of G2M checkpoint. Moreover, we also propose that the maintenance of the G2M checkpoint may not be dependent on DNA end resection, at least not in the absence of NBS1.

## 5. Materials and Methods

### Cell culture

U2OS, MRC5, HeLA and HEK293T cells were grown in Dulbecco's modified Eagle medium (DMEM, Gibco), supplemented with 10% fetal calf serum (FCS, Gibco), 100 units/ml each of penicillin and streptomycin. U2OS Flp-In T-REx cells were grown in the DMEM medium supplemented with 10% fetal calf serum (FCS, Gibco), 100 U/ml penicillin/streptomycin, 50 μg/ml hygromycin B and 10 μg/ml blasticidin S.

### Generation of stable GFP-CtIP cell lines

To generate a siRNA based complementation system for NBS1; the Flp-In T-REx system (Invitrogen, Life Technologies) was used to generate cell lines that express different siRNA-resistant NBS1myc variants in an inducible manner. The NBS1myc harboring pcDNA5/FRT/TO vector and the Flp recombinase expression plasmid pOG44 were mixed (1:9 ratio) and transfected into 80-90% confluent 6 cm dish of Flp-In T-REx 293 using Lipfectamine-2000 (Invitrogen, Life Technologies). After 6 h, the transfection reagent was removed completely and the cells were supplemented with fresh DMEM medium. After 24 h, cells were replated at different dilutions in 10-cm plates. Later after 24 h 250 μg/ml of hygromycin B and 10 μg/ml of blasticidin S. was added to the medium. The medium was replaced every 3–4 days, and cells were selected for approximately 3 weeks. Resistant colonies were picked, expanded and then characterized for inducible NBS1myc expression both by immunofluorescence microscopy and immunoblotting. To induce the expression of NBS1myc, cells were treated with 0.5 or 1 μg/ml doxycycline (DOX) for 24 h or 12 h as indicated.

### Immunofluorescence Microscopy

U2OS cells were cultured on glass coverslips and, if indicated, were irradiated at 24 h after plating. After treatment, cells were fixed in 4% formaldehyde for 12 min at room

temperature. After washing twice with PBS, cells were permeabilized with 0.25% Triton X-100 in PBS for 5 min at room temperature. After washing twice with PBS, cells were blocked with 5% FCS in TBST buffer for 30 min. Cells were then stained with rabbit NBS1, mouse myc, rabbit, cyclin A, RPA antibodies, diluted in PBS, 0.05% Tween-20, 2.5% BSA for 1 h at room temperature. After washing thrice with PBST, cells were stained with Alexa488-, Alexa568-, or Alexa647-conjugated secondary antibodies for 30 min at room temperature and counterstained with DAPI. Images were obtained using a Leica DM6000B microscope, equipped with 63×-immersion lens (PL-S-APO, numerical aperture: 1.30) and Xenon light source using LAS-AF software (Leica).

For the counting of the foci, the macro used is mentioned below:

```
n = roiManager("count");
for (i=0; i<n; i++)
{
 roiManager("select", i);
 run("Analyze Particles...", "size=0.00-infinity show=Nothing display exclude clear summarize");
 filename = "/User/ResultsMacro/Results" + i + ".xls";
 saveAs("Results", filename);
}
```

**Clonogenic and short-term survival assays**

For clonogenic survival U2OS Flp-In T-REx cell lines were transfected with siNBS1 for 48 h and then plated in triplicates at clonal density, DMEM medium supplemented with 0.5 μg/ml doxycycline. 24 h later, the cells were exposed to 1, 2, or 4 Gy of IR. After 12-14 days, colonies were fixed and stained using methanol/acetic acid/water in a 5:2:3 ratio, supplemented with 0.01% Coomassie brilliant blue and the number of surviving colonies for each cell line was tabulated. Percentage Survival for each cell type was calculated using the plating efficiencies with non-irradiated cells of each cell line as a reference. Survival experiments were carried out on at least thrice in mutant and control U2OS Flp-In T-Rex cell lines.

**Western blotting**

Total cell lysates were prepared in Laemmli sample buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol). Alternatively, cell extracts were prepared in the following buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM MgCl2, 1% NP-40, and 1 mM EDTA) supplemented with phosphatase and protease inhibitors and incubated for 30 min at 4°C. Lysates were cleared by centrifugation at 14,000*g* for 15 min and protein concentration was measured using Bradford protein assay. Immunoprecipitations were done using Anti-Flag M2 affinity gel (Sigma, A2220), Monoclonal anti-HA, A2095, Dynabeads M-280 Streptavidin (Invitrogen, 112.05D). Proteins were resolved by SDS–PAGE and transferred to nitro- cellulose. Membranes were blocked in 5% non-fat dry milk in 0.1% TBST (1xTBS supplemented with 0.1% Tween-20) for at least one hour and incubated with primary antibodies over night at 4°C in blocking buffer. Membranes were washed 3 times with 0.1% TBST, 10' each and secondary antibodies were added for 1h at room temperature. Membranes were again washed 3 times with 0.1% TBST, 10' each and detected with ECL detection reagent (GE healthcare).

Antibodies used in this study were: mouse γH2AX (Millipore, JBW302, 1:500), rabbit NBS1 (Novus Biologicals, NB100-143, 1:500), mouse NBS1 (Genetex, Clone 1D7, GTX70224, 1:500), rabbit HA (Abcam, ab9110, 1:2,000), rabbit Flag (Sigma, F7425, 1:1,000), rabbit SMC1 (Abcam, ab9262, 1:500), mouse Mcm7 (Santa Cruz, DCS141, sc65469, 1:500), mouse GFP (Roche, 11814460001, 1:1,000), mouse tubulin (Sigma, DM1A, T6199, 1:2,000), mouse Mre11 (Abcam, 12D7, ab214, 1:500), sheep MDC1 3835 (gift from S. Jackson, 1:2,000), mouse CtIP (Santa cruz, sc-271339, 1:250), rabbit pRPA32 (S4/S8) (Bethyl, A300-245A, 1:300), mouse BrdU (Amersham/GE healthcare, BU-1, RPN202, 1:100), rabbit Cyclin A (Santa Cruz, H-432, MAB3400, 1:100), rabbit pChk1 S345 (Cell Signaling, 133D3, 1:500), rabbit pChk1 S317 (Cell Signaling, 2665, 1:500), mouse BRCA1 (Santa Cruz/ LabForce, D-9, sc-6454), 1:100), rabbit Rad17 (Santa Cruz, sc-5613, 1:500), mouse Myc (GeneTex, 9E10, GTX80249, 1:250), rabbit Chk1 (), rabbit pRPA S33 (Bethyl, A300-246A, 1:250), RPA32 (Abcam, 9H8, ab2175, 1:250) rabbit

pATM S1981 (Epitmics, YE070901r, 1:5000), rabbit ATM (Calbiochem, PC-116, 1:250), mouse RAD50 (Gene Tex, 13B3, GTX70228, 1:1000)

**Generation of DNA damage**

Prior to laser irradiation, cells were grown on coverslips in cell culture dishes in the presence of 10 μM BrdU (Bromodeoxyuridine; Sigma) for 24 h. Coverslips were transferred into LabTek chamber slides (Nunc) and mounted on the microscope stage for irradiation. The MMI CELL TOOLS software with MMI UV CUT plug-in assisted the laser operation using an energy output of 50% (Larsen et al., 2014). After irradiation, cells were placed back in the incubator for 30-60 min before fixation. To generate DNA damage in subnuclear volumes, cells were irradiated with X-Rays, using YXLON.SMART 160E – 1.5 device machine (150 kV, 6 mA; YXLON International A/S), delivering 11.8 mGy $s^{-1}$. Soft X-rays were largely filtered out with a 3 mm aluminum filter. The laser microirradiation was performed with a MMI CELLCUT system containing a 355 nm UVA laser (55 Hz, Molecular Machines & Industries, Switzerland) embedded in an Olympus IX71 microscope station and focused through an LUCPLFLN 40X objective.

**Checkpoint analysis**

Cells were harvested at 1 h after IR and fixed in 70% ethanol at -20°C overnight. After fixation, the cells were resuspended in 1 ml of 0.25% Triton X-100 in PBS and incubated on ice for 15 min. After centrifugation, the cell pellet was suspended in 100 μl of PBS containing 1% bovine serum albumin (BSA) and 0.75 μg of a polyclonal antibody that specific recognizes the phosphorylated form of histone H3 (Histone H3 pS10, rabbit, Millipore, 1:200) and incubated for 2 h at room temperature. The cells were then rinsed twice with PBS containing 1% BSA and incubated with FITC-conjugated goat anti-rabbit immunoglobulin G antibody diluted at a ratio of 1:30 in PBS containing 1% BSA. After a 30-min incubation at room temperature in the dark, the cells were stained with PI and data were acquired with a flow cytometer (FC500; Becton Dickinson).

**Nocodazole trap Experiments:**

Cells were either left untreated or irradiated (4 Gy) and then either collected at 2 h after IR treatment or treated with nocodazole (50 ng/ml). At 6 h, after nocodazole treatment, the cells were collected and analyzed for H3-S10p–positive cells by flow cytometry, as described previously (checkpoint analysis)

**HR and NHEJ DNA repair assay**

DSB repair efficiency by HR was measured in DR-GFP U2OS cell lines previously (Bennardo et al., 2008). Briefly, 20,000 cells/cm$^2$ were plated in 6-well plates (poly-L-lysine coated) and, after 24 h, cells were transfected with 3 μl of 40nM siRNA oligos. The next day, siRNA treated cells were reseeded in 12-well plates, so that they are around 70-80% confluent the next day. 24 h later (48 h after siRNA transfection), cells were either mock-transfected or transfected with 0.6 μg I-SceI expression plasmid (pCBASce) in with 0.2 μg of using 2 μl of Lipfectamine-2000 (Invitrogen, Life Technologies). At 4 h after plasmids transfection, media were replaced and a second transfection with 1 μl of 40nM siRNA oligos was performed using Lipofectamine RNAiMAX. At 48 h after I-SceI transfection, cells were analyzed for GFP expression by flow cytometry on LSRII Fortessa (BD).

**DNA resection quantification by Flow cytometery**

U2OS Flp-In T-REx cell lines were seeded in 35 mm dishes, so that they are 50% confluent the next day. Cell lines were then transfected with siNBS1 and 24 h later splitted and reseeded so that they are around 80% confluent after 48 (72 h after siNBS1 treatment). Cells were then pulse-labeled with 10 μM EdU 30 min before IR treatment (10 Gy). Cells were harvested by trypsinization, 3 h after IR treatment, washed 1x with PBS and pre-extracted for removal of non-chromatin bound RPA2 by resuspending pelleted cells in 100 μl of PBS-T and incubating for 10 min on ice. After pre-extraction

cells were washed with 2 ml of 1x PBS containing 1 mg/ml of bovine serum albumin (PBS/BSA). Cells were fixed with 4% paraformaldehyde in 1xPBS for 15 min and permeabilized in 1x PBS containing 0.2% Triton X-100 for 30 min). After permeabilization cells were washed twice with 1 ml of PBS/BSA and suspended in 50 μl of PBS/BSA supplemented with the RPA antibodies (1:300). After at least 2 h incubation at room temperature, cells were washed with 2 ml of PBS/BSA. Cell pellets were then resuspended in 50 μl of PBS/BSA with the Alexa488- conjugated secondary antibodies, and incubated for 30 min at room temperature in the dark. EdU detection reaction was carried out at this point. After washing with 2 ml of 2 ml of PBS/BSA, cells were resuspended in 0.5 ml of 1x PBS containing 0.02% sodium azide, 250 μg/ml RNase A and 2 μg/ml of 40,6-diamidino-2-pheny-lindole (DAPI), then incubated at 37°C for 30 min in the dark. cells were analyzed DNA end resection by flow cytometry on LSRII Fortessa (BD).

**DNA plasmids and RNA interference:**

Plasmids were transfected in HEK-293 cells by the standard calcium phosphate method. The epitope-tagged expression vectors for human CtIP were kind gift from Sartori lab and have been described previously (Sartori et al, 2007). All NBS1 and CtIP Point mutations were introduced by using the QuickChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX, USA). For transient siRNA experiments RPE-1, MRC5, HeLa, HEK-293 or U2OS cells were plated in 6-well plates and transfected with the indicated amounts of siRNA oligos (40 nM) using Lipofectamine RNAiMAX, following manufacturer's guidelines (Invitrogen, Life Technologies). In brief, medium was replaced with Opti-MEM (Gibco, Life Technologies) prior to incubation with siRNAs and Lipofectamine. After 12 h, medium containing FCS (10% final concentration) was added and cells were analyzed at 72 h after siRNA transfection.

**siRNA and mutagenesis primers**

The siRNA oligonucleotides were obtained from Microsynth AG (Balgach, Switzerland). The sense strand sequences of all siRNAs used, are the following:

NBS1 #1: 5'-GGAGGAAGAUGUCAAUGUUTTdTdT-3' (Larsen et al., 2014)

NBS1 #2: 5'-CCAACUAAAUUGCCAAGUATTdTdT-3' (Larsen et al., 2014)

CtIP: 5'-GCUAAACAGGAACGAAUCTTdTdT-3' (Sartori et al, 2007)

MDC1: 5'-GUCUCCCAGAAGACAGUGAdTdT-3'

Rad17: 5'- CAGACUGGGUUGACCCAUCdTdT-3' (Wang et al., 2014b),

BRCA1: 5'-GGAACCUGUCUCCACAAAGTTdTdT-3'

For site directed mutagenesis primers (PAGE purified), were also ordered from Microsynth AG (Balgach, Switzerland). The site directed mutagenesis PCR was performed as described in the manual provided with mutagenesis kit (Agilent Technologies, # 200516). The sense strand sequences of all DNA oligonucleotides used, as mutagenesis primers are the following:

NBS1 siNBS1 #1:

5'- GTTCAAAAACAGGAGGAAGACGTGAACGTTAGAAAAAGGCCAAGG-3'

NBS1 S432A:

5'-CCAAACTATCAGCTTGCACCAACTAAATTGCCA-3'

NBS1 R28A:

5'-GAGTACGTTGTTGGAGCGAAAAACTGTGCC-3'

NBS1 K160M:

5'-GGTATCAGTGAAAGTTACCATTATGACAATATGTGCACTCATTTGTGG-3

CTIP T671A:

5'-CTCAGTATAAAATCGTTGCTGTAATAGATACAAAGGATG-3'

CTIP T687A:

5'-CAAAATTAGGAGGAGAGGCAGTGGACATGGAC-3'

CTIP T698A

5'-CTGTACATTGGTTGCTGAAGCCGTTCTCTTAAAAATG-3'

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| 2011 - Present | ***Ph.D. in Cancer Biology***<br>University of Zurich, Switzerland<br>**Project title:** Role of NBS1 in DNA double-strand break response |

**Honors, scholarships, prizes and awards**

| | |
|---|---|
| CSIR-JRF | June 2009, (All India Rank 163) |
| CSIR-NET | December 2009 |
| GATE 2009 | Percentile 98.05, (All India Rank 245) |
| GATE 2010 | Percentile 98.50, (All India Rank 147) |
| UoH Entrance test | 2009, All India top ranker in the entrance exam for M.Tech |
| Travel award 2015 | From Cancer Biology PhD Program to attend the EMBO's conference on “The DNA damage response in cell physiology and disease” |

**Conferences and Presentations**

Cancer Network Retreat, October 2011, Grindelwald, Switzerland, *poster presentation*: “Functional implications of DNA damage induced MDC1 dimerization”.

Cancer Biology PhD Program Retreat, February 2014, Filzbach, Switzerland, *oral presentation*: “Role of NBS1 in DNA end resection”.

Replication, recombination and repair club, Cancer Network Zurich, Switzerland, April 2014, *oral presentation*: “Role of NBS1 in DNA end resection”.

Swiss Meeting on Genome Stability and Chromatin Dynamics, Weggis, Switzerland, May 2014, *poster presentation*: “Role of NBS1 in DNA end resection”.

Joint Cancer Meeting, Cancer Network Zurich, June 2015, *oral presentation*: “Exploring the role of tumor suppressor protein NBS1 in DNA double strand break repair”.

EMBO conference “The DNA damage response in cell physiology and disease”, Greece, October 2015, *poster presentation*: “Role of NBS1 in DNA end resection”.

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